Radioimmunoassay for Pregnancy-Associated Plasma Protein A

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A specific and highly sensitive radioimmunoassay for determination of pregnancy-associated plasma protein A in human serum is described. The minimum detection limit for this protein was 2.9 μg/L. The within- and between-assay coefficients of variation were 4.0 and 4.5%, respectively. The circulating protein was detected within 32 days of conception in eight normal pregnancies and within 21 days in a twin pregnancy. Circulating concentrations in the mother at term were consistently higher (10-fold) than in matched amniotic fluid; none was detected in the umbilical circulation. This protein was also detected in the circulation of patients with hydatidiform mole. This assay will permit investigations into the clinical evaluation of measurements of the protein during early pregnancy and trophoblastic disease.

Additional Keyphases: trophoblastic tumors • cancer • fetal status • amniotic fluid • measurements during the first trimester of pregnancy

The human trophoblast produces a wide range of specific proteins, which are primarily secreted into the mother’s circulation. Measurement of some of these molecules is widely applied in the diagnosis of pregnancy and as an index of fetal well-being.

Pregnancy-associated plasma protein A (PAPP-A) is one of four pregnancy-specific molecules recently described by Lin et al. (7). Although some biochemical characteristics of this protein have been described, its biological function is unknown. PAPP-A is a glycoprotein of high molecular mass (750,000 daltons), with alpha-2 electrophoretic mobility. Although concentrations of circulating PAPP-A are readily measured in advanced pregnancy by electroimmunoassay (2), there is no information on PAPP-A during the first half of the pregnancy. We describe here a highly sensitive radioimmunoassay for PAPP-A, which we have applied to serum obtained from normal and abnormal first-trimester pregnancies, maternal and fetal serum, and amniotic fluid obtained at term delivery.

Materials and Methods

Materials

Purified PAPP-A and standards: PAPP-A used for radioimmunoassay and standardisation was purified by a two-step immunoaffine affinity chromatographic procedure (3, 4). Pooled late-pregnancy serum (LPS) was obtained from 50 volunteers (30 to 40 weeks of gestation) and calibrated against this purified preparation. Purified PAPP-A and aliquots of the reference material were stored at -20 °C.

Antiserum to PAPP-A: The rabbit anti-human PAPP-A antisera used in this study (3) demonstrated similar specificity and dose-response characteristics as antisera kindly supplied by P. Bischof, University of Geneva, Switzerland. PAPP-A antisera were stored in aliquots at -20 °C.

Procedures

Radioiodination of PAPP-A: Purified PAPP-A was labeled with 125I by the method of Bolton and Hunter (5). We incubated 7 μg of PAPP-A in 12 μL of phosphate buffer (50 mM, pH 8.0) with 1 mCi of N-hydroxysuccinimide ester of [125I]hydroxyiodophenylpropionic acid at 4 °C for 12 h. Then we added 500 μL of 0.2 mol/L glycine in phosphate buffer (50 mM, pH 8.0) while thoroughly stirring, and allowed the mixture to incubate at room temperature for 5 min. Undamaged 125I-labeled PAPP-A was separated from damaged material and unreacted iodide by chromatography on a 18 × 1.2 cm column of Sephadex G-200 (Pharmacia, Uppsala, Sweden) equilibrated with the pH 8.0 phosphate buffer containing 2.5 g of gelatin per liter. Fractions of 500-μL volume were collected and assessed for immunoreactivity.

Assessment of immunoreactivity: Appropriate fractions containing undamaged 125I-labeled PAPP-A were assayed for immunoreactivity by overnight incubation of 200 μL of tracer (15,000 cpm) in an excess of antibody to PAPP-A (200 μL at 500-fold initial dilution) at room temperature.

Separation of bound and free antigen: To separate bound and free antigen, we added donkey antisera to rabbit immunoglobulins (Wellcome Reagents, Sydney, Australia) and polyethylene glycol 6000 (BDH Chemicals (Australia) Pty. Ltd., Port Fairy, Australia). The optimum dose of polyethylene glycol 6000 used was that which gave minimum nonspecific binding and approached maximum binding (see Figure 1).

Titration of antibodies to PAPP-A: Serially diluted antiserum (200 μL) was added to antigen-free and antigen-containing tubes containing 200 μL of tracer (15,000 cpm). The mixture was incubated overnight at room temperature, and free and bound tracer were separated as described above.

Radioimmunoassay procedure: The final incubation volume was 500 μL. All dilutions were made with phosphate-buffered (50 mM, pH 7.5) isotonic saline containing 1 g of bovine serum albumin per liter. We incubated 100 μL of sample or standard, 200 μL of antibody to PAPP-A (20,000-fold initial dilution), and 200 μL (15 000 cpm) of 125I-labeled PAPP-A overnight at room temperature. Samples containing high concentrations of PAPP-A were diluted in serum from males. Bound and free antigen were separated by precipitation by adding 100 μL of second antibody (20-fold initial dilution) and 1 mL of polyethylene glycol 6000 (75 g/L) and centrifuging at (1500 × g, 4 °C, 40 min). The supernate was discarded and the radioactivity in the pellets was counted.

Sample Collection

Sera. Control group: Venous blood was obtained from 100 essentially normal volunteers (both sexes) who were undergoing elective surgery for non-malignant conditions.

Pregnancy group: From 12 women who were attending the infertility clinic we obtained venous blood at frequent intervals from the time of ovulation. The time of ovulation was determined by history; changes in the cervical mucus, basal
body temperature, and plasma estradiol; and ovarian ultrasonic measurements. Matched maternal and cord arterial and venous blood and amniotic fluid were obtained from eight women who were undergoing elective cesarean section because of suspected disproportion.

Miscellaneous: Venous blood was obtained from two patients with hydatidiform mole, one with choriocarcinoma, and one with endodermal sinus tumor, before treatment. Serum was separated within 2 h of collection and stored at −20 °C until assay.

Placental homogenate. Placental tissue was obtained from a term normal pregnancy and homogenized (6). The supernate was stored at −20 °C until assayed.

Results

Analytical Variables

Iodination: Approximately 20% of the added 125I was incorporated into the PAPP-A molecules, resulting in a specific activity of 14 Ci/g of PAPP-A, i.e., five 125I atoms per PAPP-A molecule. Gel filtration of the reaction mixture resulted in two major peaks (Figure 2): undamaged labeled protein in the first peak and unreacted iodide in the second.

Fractions 8, 9, and 10 from the elution profile (Figure 2) were used in the assay. These fractions showed a binding of 60–70% in excess of PAPP-A antibody and an assay blank value of <12%.

PAPP-A antibody dilution curve: At the initial 20 000-fold dilution of antiserum, 45 to 50% of the tracer was bound. We used this concentration in preparing the standard curve.

Standard curve: Calibration of a pool of late-pregnancy serum against the purified PAPP-A revealed that this pool had a PAPP-A concentration of 47 mg/L. The standard curve was linear with concentration from 5.6 to 1466 μg/L, and the minimum detection limit (i.e., the minimum amount of PAPP-A that could be statistically distinguished from zero standard at 2.0 SD) was 2.9 ± 0.8 μg/L. This standard curve was used for all assays and precision studies. The within-assay imprecision (CV) was between 2.5 and 4.0% (n = 40), the between-assay CV between 4.1 and 4.5% (n = 8).

Statistical analysis of the standard curve showed that the response–error relationship ranged from 0.00 to 0.04. The CV profile across the standard curve was a parabola with peak values of 7.2 and 7.4% at the minimum and maximum PAPP-A doses, respectively [i.e., at (% B/B0) of 95 and 20%]. The nadir of the parabola was between 2.7 and 2.9% at (% B/B0) of 75 and 65%. These standard-curve characteristics are demonstrated by its ±2.0 SD error envelope (Figure 3).
**Specificity:** No cross reaction was observed with a substantial molar excess of pregnancy-specific β₁-glycoprotein (40 mg/L), alpha-fetoprotein (40 mg/L), placental protein 5 (40 mg/L), human chorionicadotropin (800 pituitary units/L), or thyroglobulin (100 mg/L). Circulating PAPP-A in concentrations exceeding the assay detection limit was not observed in any of 100 non-pregnant normal volunteers whom we studied.

**Clinical Studies**

Serial dilutions of purified PAPP-A, late-pregnancy serum, serum from a patient with hydatidiform mole, and the supernatant fluid from a placental homogenate yielded parallel dose–response curves (Figure 4).

Circulating concentrations of PAPP-A were detected between 28 and 32 days after ovulation in the case of singleton pregnancies and 21 days after ovulation in a twin pregnancy. Concentrations increased exponentially thereafter (Figure 5). PAPP-A was not detected (<3.3 μg/L) in the circulation of two patients with spontaneous miscarriage (blood sampled on days 32 and 44), or one patient with tubal gestation (sampled on day 42). The mean concentration of PAPP-A in the mothers' sera in term pregnancy was 36.07 (SD 22.04) mg/L. PAPP-A was detected in all amniotic fluids examined, but was ≤7.4% of the concentrations found in the respective mothers' sera. In contrast, PAPP-A was not detected in the umbilical circulation. Circulating PAPP-A was also detected in two patients with hydatidiform mole (150 μg/L; 15.04 mg/L) but not in the patient with choriocarcinoma or the one with endodermal sinus tumor.

**Discussion**

Since the original identification of this new protein in the plasma of pregnant women, the scarcity of purified preparations of PAPP-A has until now prevented the development of specific and sensitive assays. Affinity-chromatographic procedures involving antisera generated from fractionated late-pregnancy blood have been used successfully for PAPP-A purification (3). More recently, a simple and rapid two-step affinity-chromatographic procedure yielding large amounts of purified antigen has been described (4). The availability of this purified antigen has resulted in the development of the sensitive and specific radioimmunoassay described here, permitting detection and precise quantification of PAPP-A during early pregnancy and in trophoblastic disease.

In developing the assay we encountered no unusual problems. Iodination was done by a standard technique, and bound and free antigen were separated by use of second antibody and polyethylene glycol. The latter technique was chosen for routine use because it is rapid and reliable. The standard curve was "targeted" to obtain maximum sensitivity for examination of samples obtained during early pregnancy and malignancy. The sensitivity of the assay could be varied by appropriate changes in the antibody concentration. Specificity was excellent for the materials examined at the maximum sensitivity of the assay. The precision characteristics of this assay compare favorably with WHO recommendations for precision, with respect to within- and between-assay variance and the response–error relationship of the standard curve (7).

Although the clinical application of this assay is in its infancy, the assay may be used equally for the quantification of circulating PAPP-A in early and late pregnancy, hydatidiform mole, amniotic fluid, and placental homogenates. Circulating PAPP-A was detected soon after conception in normal pregnancy, but not in patients with spontaneous miscarriage. PAPP-A—like other placental products such as placental lactogen and pregnancy-specific β₁-glycoprotein—appears to be secreted unidirectionally into the maternal circulation; only small amounts are detectable in amniotic fluid. This molecule was also detected in the circulation of patients with hydatidiform mole. These results suggest that PAPP-A measurement may be an index of trophoblastic function, and that further studies into the function and clinical usefulness of this protein are warranted.

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Fig. 4. Standard dose–response curves for purified PAPP-A (■) as compared with PAPP-A in (X) maternal serum, (○) placental homogenate, and (▲) plasma from a patient with hydatidiform mole.

Fig. 5. Circulating concentrations of PAPP-A in early pregnancy, demonstrating the time of first detection and the exponential increase of PAPP-A. ▲ singleton pregnancy; • twin pregnancy.
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References