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Pregnancy Specific β_1 Glycoprotein: A Comparison of Analysis by Radioimmunoassay and Enzyme Linked ImmunoSorbent Assay Observations on Patients with Malignant Teratoma of Testis

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Summary: A comparison of serum pregnancy specific β_1 glycoprotein analysis by enzyme linked immunosorbent assay and radioimmunoassay has been made. Excellent correlation was obtained between the two methods but radioimmunoassay was found to be more precise and to have a lower detection limit than enzyme linked immunosorbent assay. Using the more sensitive radioimmunoassay system, 2 out of 10 control male sera were found to contain detectable levels of pregnancy specific β_1 glycoprotein. Sequential results are presented on patients with diagnosed malignant teratoma of testis who were undergoing chemotherapy.

Schwangerschaftsspezifisches β_1 -Glykoprotein:

Vergleich der Analyse mit Radioimmunassay und Enzyme Linked ImmunoSorbent Assay.

Beobachtungen an Patienten mit malignen Hodenteratomen

Zusammenfassung: Ein Vergleich der Analyse von schwangerschaftsspezifischem β_1 -Glykoprotein mit Enzyme Linked ImmunoSorbent Assay (ELISA) und Radioimmunassay wurde durchgeführt. Eine hervorragende Korrelation zwischen beiden Methoden wurde erhalten, jedoch zeigt der Radioimmunassay eine bessere Zuverlässigkeit und hat eine niedrigere Nachweisgrenze als der Enzyme Linked ImmunoSorbent Assay. Mit Hilfe des empfindlicheren Radioimmunassay wurden in zwei von zehn Kontrollseren von Männern nachweisbare Konzentrationen von schwangerschaftsspezifischem β_1 -Glykoprotein gefunden. Fortlaufende Ergebnisse bei Patienten mit diagnostizierten malignen Hodenteratomen unter Chemotherapie werden mitgeteilt.

Introduction

Pregnancy specific β_1 glycoprotein is produced by the syncytiotrophoblasts during pregnancy, when peripheral blood concentrations greater than 400 mg/l may occur during the last trimester (1). Additionally pregnancy specific β_1 glycoprotein may be produced by trophoblastic tumours in both men and women but under these circumstances, serum concentrations are 1/1000 of those found in pregnancy (2). Serum pregnancy specific β_1 glycoprotein has been quantitated by radial immunodiffusion (3), radioimmunoassay (2) and more recently enzyme linked immunosorbent assay (1). Enzyme linked immunosorbent assay has several advantages over radioimmunoassay (4) so we wished to apply the technique to serum pregnancy specific β_1 glycoprotein analysis in malignancy. To assess the suitability of the technique for this purpose, we have undertaken a comparison with an estab-

lished radioimmunoassay for pregnancy specific β_1 glycoprotein (2).

Materials and Methods

Reagents

1) Pregnancy specific β_1 glycoprotein standards – Hoechst Pharmaceuticals, Hounslow, England – A range of dilutions was prepared in a serum pool composed of blood from male and non-pregnant female volunteers.

2) Pregnancy specific β_1 glycoprotein antiserum – Dako Immunoglobulins Ltd., Copenhagen. Supplied by Mercia Brocades. Diluted 1:1200 in carbonate-hydrogencarbonate buffer (pH 9.6) before use.

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3) Antibody-enzyme conjugate – Dako Immunoglobulins Ltd., Copenhagen. Diluted 1:500 in phosphate buffered saline/chicken serum before use.

4) Iodinated pregnancy specific β_1 glycoprotein.

5) Bovine serum albumin – Hoechst Pharmaceuticals, Hounslow, England.

6) Chicken serum – Flow Laboratories, Irvine, Scotland.

7) Microtitre plates – M 129A Immulon. Dynatech Laboratories. Supplied by Gibco Biocult, Paisley, Scotland.

8) Chromogen-o-Phenylene diamine. Sigma London Chemical Co. Ltd., Poole, England.

Clinical material

Serum samples were obtained from 29 patients with disseminated malignant teratoma of testis. Twelve patients had raised serum chorionic gonadotropin concentrations and of these, ten had concurrently raised serum α -foetoprotein. Serial results from two selected patients undergoing chemotherapy are presented. Blood was collected from 10 healthy male laboratory staff to comprise a control group. Prior to assay all sera were heated at 56 °C for 30 minutes to destroy the complement system which is thought to interfere with enzyme linked immunosorbent assay (5). Preliminary investigations have shown that pregnancy specific β_1 glycoprotein is not destroyed by this procedure.

Assay Procedures

Radio Immuno Assay (RIA)

Serum or standards (100 μ l) were mixed together with 100 μ l of antiserum (previously diluted 1:50000 in phosphate buffered saline containing 10 g/l bovine serum albumin). Iodinated pregnancy specific β_1 glycoprotein was diluted in phosphate buffered saline to give about 20000 counts per minute per 100 μ l. 100 μ l of this dilution was added to the antigen/antibody mixture and incubated for 24 hours at room temperature. The bound fraction was precipitated overnight at +4 °C using a double antibody technique (normal rabbit serum 1/200 and donkey antirabbit serum 1/32). After centrifuging for 45 minutes at 2000 min^{-1} using a 290 mm radius head in a Mistral 6L centrifuge, the supernatant was aspirated and the bound fraction counted. A graph of bound/total counts against concentration was constructed and used to calculate the concentrations of pregnancy specific β_1 glycoprotein in the test sera.

Enzyme Linked ImmunoSorbent Assay (ELISA)

The method used was that of Macdonald et al. (1), but to achieve maximum sensitivity, incubation times were increased to 60 minutes and colour reaction to 45 minutes. Serum samples (100 μ l) were used undiluted and a commercial antibody-enzyme preparation now available was used at a dilution of 1/500.

Developed colours were read on an Abbott ABA 100 bichromatic analyzer.

Comparison of assay characteristics

Precision

Within batch precision was determined at two concentrations from 24 (enzyme linked immunosorbent assay) or 20 (radioimmunoassay) replicates. Overall precision was derived from duplicate analyses in each of 12 (enzyme linked immunosorbent assay) or 8 (radioimmunoassay) assays.

Detection limits

Detection limits of both radioimmunoassay and enzyme linked immunosorbent assay was defined in terms of the standard deviation of duplicate counts or absorbance difference values for a 0 μ g/l standard, according to the formula:

$$\text{Standard Deviation} = \sqrt{\frac{D^2}{2(N-1)}}$$

where D is the difference between duplicate counts for 0 μ g/l standard (B_0) in the radioimmunoassay,

or

where D is the difference in duplicate counts for 0 μ g/l standard. N is the number of duplicates.

For radioimmunoassay the detection limit was defined as the concentration of pregnancy specific β_1 glycoprotein corresponding to $B_0 - 2$ standard deviation, i.e. that concentration could be distinguished for 0 μ g/l with 95% probability. For enzyme linked immunosorbent assay, the detection limit was defined as the concentration of pregnancy specific β_1 glycoprotein corresponding to absorbance of 0 μ g/l standard + 2 standard deviation.

Results obtained by the two methods

Serum pregnancy specific β_1 glycoprotein concentrations obtained by both methods were compared using standard statistical formulae (6). To obtain a valid comparison, the standards used in both assay systems were prepared from the same stock. The same antiserum was also used in both systems.

Specificity of Antiserum

α -Foetoprotein and human chorionic gonadotropin were both checked for cross reaction in the radioimmunoassay system.

Results

Figure 1 demonstrates the specificity of the antiserum. The precision of both assay systems is given in tables 1 and 2. Detection limits were assessed over 5 standard curves for each assay and a mean value taken. Radioimmunoassay had a detection limit of 1.44 μ g/l and enzyme linked immunosorbent assay a detection limit of 3.6 μ g/l. Table 3 shows the statistical analysis of the comparison between the two assay systems.

Fig. 1. Cross reactivity of chorionic gonadotropin and α -foetoprotein in the radioimmunoassay of pregnancy specific β_1 glycoprotein.
 ▲—▲ Pregnancy specific β_1 glycoprotein – standard curve.
 ●—● Chorionic gonadotropin
 ■—■ α -foetoprotein

Tab. 1. Precision of pregnancy specific β_1 glycoprotein determination by radioimmunoassay. Statistics are based on 20 replicate analyses for within batch precision or duplicate analyses from 8 assays for overall precision.

	Mean	Standard deviation	Coefficient of variation for duplicate analyses
	($\mu\text{g/l}$)	($\mu\text{g/l}$)	(%)
a)			
Within batch	3.44	0.40	11.5
Overall	2.38	0.23	11.6
b)			
Within batch	106.4	9.4	8.8
Overall	97.4	9.1	9.3

Tab. 2. Precision of pregnancy specific β_1 glycoprotein determination by enzyme linked immunosorbent assay. Statistics are based on 24 replicate analyses for within batch precision or duplicate analyses from 12 assays for overall precision.

	Mean	Standard deviation	Coefficient of variation of duplicate analyses
	($\mu\text{g/l}$)	($\mu\text{g/l}$)	(%)
a)			
Within batch	3.03	0.46	15.2
Overall	2.70	0.52	19.4
b)			
Within batch	98.5	12.8	13.0
Overall	94.2	12.9	13.6

Tab. 3. Regression statistics for pregnancy specific β_1 glycoprotein determined by radioimmunoassay and enzyme linked immunosorbent assay.

Correlation coefficient	0.98
Number of pairs of observations	18
Gradient	0.90
Y axis intercept	0.24
Standard error of gradient	0.029
Standard error of intercept	0.31
Pair difference t test	1.7*)

*) not statistically significant at $p > 0.05$

None of the control group had serum concentrations greater than $3.6 \mu\text{g/l}$ when pregnancy specific β_1 glycoprotein was measured by enzyme linked immunosorbent assay. When radioimmunoassay was used, a range of $0-1.9 \mu\text{g/l}$ was obtained.

The results of several measurements made on serum from two patients with teratoma of the testes are shown in figure 2.

Fig. 2. Time course of pregnancy specific β_1 glycoprotein in two typical patients.

▲—▲ Patient 1
■—■ Patient 2 (remains marker negative and well)

Discussion

Neither the bound antibody-antigen, the bound antigen-antibody enzyme, nor the colour reactions proceeded to equilibrium. Thus the timing of each reaction is critical. To allow the completion of analysis within the working day, the two antibody-antigen reaction were allowed to proceed for 60 minutes and the colour development for 45 minutes. There was no cross reaction with either chorionic gonadotropin or α -foetoprotein in the radioimmunoassay system and so both assay systems may be used to measure pregnancy specific β_1 glycoprotein in the presence of chorionic gonadotropin and α -foetoprotein. At both concentrations considered, radioimmunoassay was more precise and also had a detection limit half that of enzyme linked immunosorbent assay. These findings are in contrast to the results of Grenner who found a detection limit of $0.35 \pm 0.05 \mu\text{g/l}$, an intraassay coefficient of variation of 6.5% and an interassay coefficient of variation of 8.5%. (7). Analyses by both methods showed a high degree of correlation ($r = 0.98$). Regression analysis indicates that radioimmunoassay gives slightly lower results than enzyme linked immunosorbent assay but this is not confirmed by the paired difference t-test.

Of the controls, only 2 out of 10 showed serum pregnancy specific β_1 glycoprotein, above the limit of detection ($1.44 \mu\text{g/l}$) for radioimmunoassay, these concentrations being $1.7 \mu\text{g/l}$ and $1.9 \mu\text{g/l}$.

There is at present discordance on the occurrence of pregnancy specific β_1 glycoprotein in the serum of a healthy non-pregnant population.

Thus Searle et al. (8) found detectable serum pregnancy specific β_1 glycoprotein in 13% of a healthy male and non-pregnant female population, using an assay with a sensitivity of $2 \mu\text{g/l}$, whilst Grudzinskas et al. (9) and Towler et al. (10) could find no detectable pregnancy specific β_1 glycoprotein in this group using diluted

serum. Grudzinskas' assay however, had a minimum detection limit of 8 $\mu\text{g/l}$. Towler using an assay with a detection limit of 1 $\mu\text{g/l}$ found that 30% of undiluted sera had detectable levels of pregnancy specific β_1 glycoprotein in the range 1–3 $\mu\text{g/l}$. Our data therefore seems in accordance with the findings of Towler and Searle.

Whether this apparent presence of pregnancy specific β_1 glycoprotein in normal sera is due to interaction of unspecified serum components in undiluted sera (11) is not known. With enzyme linked immunosorbent assay having a higher detection limit, no normals had detectable levels of serum pregnancy specific β_1 glycoprotein. Whether this is due to the higher detection limit or whether undiluted sera do not interact in this form of assay is not yet determined.

The two patients shown are typical of those patients having detectable pregnancy specific β_1 glycoprotein. Of the 29 patients studied all except one were marker positive (chorionic gonadotropin, α -foetoprotein or pregnancy specific β_1 glycoprotein) at some time during the period of study. Eleven patients showed elevated concentrations of pregnancy specific β_1 glycoprotein in conjunction with increased chorionic gonadotropin levels whilst only 3 patients with α -foetoprotein increases and no concurrent chorionic gonadotropin increase showed increased concentrations of pregnancy specific β_1 glycoprotein. Eleven patients showed increased α -foetoprotein concentrations alone and two patients were only pregnancy specific β_1 glycoprotein positive. One patient remained marker negative and one patient showed only chorionic gonadotropin increase. Although chorionic gonadotropin concentrations in these patients varied widely, pregnancy specific β_1 glycoprotein concentra-

tions seem to follow the same time course as chorionic gonadotropin.

This study was unable to demonstrate that pregnancy specific β_1 glycoprotein could be a more sensitive diagnostic index than chorionic gonadotropin, as patients were selected on grounds of a raised chorionic gonadotropin. A survey involving assay of pregnancy specific β_1 glycoprotein on patients diagnosed histologically alone is required. As with Lange's series (12) we found elevated pregnancy specific β_1 glycoprotein levels more frequently in conjunction with raised chorionic gonadotropin levels than with raised α -foetoprotein.

A larger and longer term survey is envisaged to consider the use of pregnancy specific β_1 glycoprotein in trophoblastic tumours of testis. Increased serum concentration of pregnancy specific β_1 glycoprotein have also been described in non-trophoblastic teratoma of testis (13) and further work is required in this area.

For assay of pregnancy specific β_1 glycoprotein as a tumour marker, an assay with a low detection limit and reasonable precision at low concentrations is required. The assay of pregnancy specific β_1 glycoprotein by radioimmunoassay fulfills these requirements.

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