The Characterization of Alpha-Subunit of Glycoprotein Hormone Produced by Undifferentiated Carcinoma

RYUICHIRO NISHIMURA¹, TAMOTSU HAMAMOTO¹,
NORIIHIKO MORIMOTO², MITSURU OZAWA²,
YOSHIHIKO ASHITAKA¹ AND SHIMPEI TOJO¹

¹Department of Obstetrics and Gynecology, Kobe University, School
of Medicine, Kobe 650, and ²Department of Obstetrics and
Gynecology, Osaka National Hospital, Osaka, Japan

Abstract

The glycoprotein hormone α -subunit was extracted and purified from the urine of a patient with undifferentiated carcinoma producing isolated α -subunit. Its final specific immunoactivity was 0.92 (mg α -subunit/mg protein). The α -subunit exhibited virtually identical immunoantigenicity to hCG- α antiserum with standard hCG- α . The molecular weight of the α -subunit determined by gel chromatography on Sephadex G-100 was greater than that of standard hCG- α dissociated by urea *in vitro*. By SDS disc electrophoresis, however, the α -subunit moved faster than hCG- α separated by mercaptoethanol reduction. The amino acid composition of the α -subunit was quite similar to that of standard hCG- α . In the isoelectric focusing, the major components of the α -subunit from undifferentiated carcinoma and the α -subunit from urine of normal pregnant women (third trimester) were distributed over the range from pH 3.5 to 6.0, while standard hCG- α was distributed in the fractions ranging from pH 6.0 to 8.0. The result of a combination study *in vitro* indicated that both α -subunits from undifferentiated carcinoma and from urine of normal pregnant women did not actively combine with hCG- β .

These results suggest that the α -subunit secreted by undifferentiated carcinoma is virtually identical with standard hCG- α as the protein moiety but differs in regard to carbohydrate moiety, and also suggest that the excess of α -subunit, which is not associated with β -subunit, may have undergone some intracellular modification, and consequently, the electric charge of the freely secreted α -subunit changes and it no longer has the ability to combine with the β -subunit.

In addition to the eutopic synthesis and secretion of free subunits of glycoprotein hormones (Franchimont and Reuter, 1972; Laburthe *et al.*, 1973; Vaitukaitis *et al.*, 1976; Nishimura *et al.*, 1979), ectopic production has been demonstrated *in vivo* (Weintraub and Rosen, 1973; Rosen and Weintraub, 1974) and *in vitro* (Tashjian *et al.*, 1973; Chou *et al.*, 1976; Liebllich *et al.*, 1976). Pioneering studies on the characterization of ectopically produced α -subunits have been reported by

the group of Weintraub (Weintraub *et al.*, 1975), who extracted and purified α -subunit from *in vivo* material to permit the amino acid analyses.

In placenta, hCG is likely synthesized *via* the association of α - and β -subunits which are synthesized from two different messenger RNAs (Boime *et al.*, 1978). Therefore, to know the characteristics of free α -subunit is worthwhile in investigating the biosynthesis of glycoprotein hormones.

In the present study, we extracted and purified α -subunit from urine of a patient with

undifferentiated carcinoma producing large amounts of isolated α -subunit, and compared its characteristics with standard hCG- α separated from hCG *in vitro* and α -subunit from urine of normal pregnant women.

Materials and Methods

Case report

A 32-year-old para 2 Japanese woman was admitted to Osaka National Hospital in September 1977 because of gradual enlargement of a left femoral tumor, which was resected in December 1977. A chest X-ray showed multiple round shadows eight months after the initial treatment. In July 1979, we were consulted concerning her amenorrhea of three months duration, and noted a positive pregnancy test. Further examination revealed the ectopic production of immunoreactive hCG- α . The lung lesions were identified with the femoral tumor by autopsy, and diagnosed histologically as undifferentiated carcinoma. The localization of hCG- α was detected by immunohistological study.

Extraction and purification of α -subunit from the urine of a patient with undifferentiated carcinoma

Extraction of urine

Urine of the patient was extracted by the standard kaolin-acetone method which had been developed as an extraction method for urinary hCG (Borth *et al.*, 1961).

All steps of extraction and purification were carried out at 4°C. Eluates from the column were monitored for protein content by measuring absorbance at 280 nm with an Hitachi 124 spectrophotometer and assayed by homologous hCG- α radioimmunoassay (Ashitaka *et al.*, 1974). Protein determinations for specific immunoactivity of α -subunit were made by the method by Lowry *et al.*, (1951).

Chromatography on DEAE-cellulose

The lyophilized powder was dissolved in 50 ml of 0.005 M Tris-HCl buffer, pH 8.0. This solution was applied to a column (2×30 cm) on DEAE-cellulose (Brown, Lot No. MOK 8299) equilibrated with a 0.005 M Tris-HCl buffer, pH 8.0. The elution was performed by a linear gradient of NaCl concentration, from 0–0.3 M, in 500 ml of the same buffer. Five ml fractions were collected at a flow rate of 15 ml/hour with a ISCO fraction collector.

Gel chromatography on Sephadex G-100

The major peak from DEAE-cellulose chromatography was passed over a Sephadex G-100 column (2.8×96 cm) in 0.05 M sodium phosphate buffer, pH 7.4. Three ml fractions were collected at a flow rate of 10 ml/hour. All fractions under the peak of the absorbance and the immunoactivity of hCG- α were pooled, dialyzed extensively against distilled water and

lyophilized.

Extraction and partial purification of α -subunit from urine of normal pregnant women

α -subunit was extracted from 50 l of urine of normal pregnant women (third trimester) using the kaoline-acetone method. Extracted material was subjected to gel chromatography on Sephadex G-100 in the same conditions as described for the purification of α -subunit from urine of the patient.

Elution profile of α -subunit from undifferentiated carcinoma on Sephadex G-100

¹²⁵I-labeled α -subunit from undifferentiated carcinoma was gel-filtrated on a 1.5×90 cm Sephadex G-100 column. The column was calibrated with blue dextran, ¹²⁵I-hCG and ¹²⁵I-hCG- α . A ISCO fraction collector was used to collect 2.5 ml fractions at 4°C at a flow rate of 12 ml/hour.

SDS disc electrophoresis

Sodium dodecyl sulfate gel electrophoresis was performed in 7.5% polyacrylamide gel as described by Weber and Osborn (1969). The samples were dissolved in 0.02 M phosphate buffer, pH 7.2, containing 1% SDS, 2% 2-mercaptoethanol, 0.05% bromophenol blue and heated at 100°C for two minutes before the electrophoretic run. For the determination of molecular weight by electrophoresis the standard molecular marker (BDH, monomer of lysozym=14,300) was applied under the same conditions.

Amino acid analysis of α -subunit from undifferentiated carcinoma

Approximately 1 mg of the α -subunit was hydrolyzed with 6 N HCl in a sealed tube at 110°C for 12 hours. After the removal of acid, the hydrolysate was analyzed by amino acid analyzer (JLC-6AH, JEOL). Tryptophan was not determined.

Isoelectric focusing

A column apparatus (Kato Co. Ltd., Japan) of 110 ml capacity was used for this study. Approximately 2 μ g of samples were introduced into the sucrose gradient from 0 to 50%. The mean concentration of ampholine (LKB) was 2%. The column was refrigerated by circulating cold ethanol using a refrigerated circulating bath (Neslab, Model RTE 5). Isoelectric focusing was carried out for 12 hours at 400 volts, followed by 40 hours at 750 volts by a power supply (MS). Fractions (1.5 ml) were then collected with a microfractionator (Gilson), and the pH of these fractions was immediately measured.

Combination of alpha- and beta-subunits

The ability of α -subunit from undifferentiated carcinoma to combine hCG- β subunit was determined by hCG-RIA. The recombination of standard hCG- α and hCG- β , separated by urea *in vitro*, was also

studied as a control. The methods of combination study and calculation of the per cent of theoretical combination at time course were performed as described by Weintraub *et al.* (1977).

Fifteen μ g of the α subunit and standard hCG- α were individually incubated with 23 μ g of hCG- β according to molecular weight. The incubation was carried out at 4°C and 27°C in 100 μ l of 0.01 M sodium phosphate, pH 7.4, in plastic tubes (5 $\times$ 20 mm) sealed with parafilm. Five μ l aliquots were removed at zero time and varying time, diluted in 2 ml of cold 0.05 M sodium phosphate, pH 7.4, containing 0.05% BSA and stored at -80°C until assay.

Results

Extraction and Purification

Table 1 shows the results of extraction and purification of α -subunit from urine of the patient with undifferentiated carcinoma. Although the kaolin-acetone standard method had been developed as an effective extraction method for urinary hCG, α -subunit could be also extracted from urine virtually by the same procedure. As the starting material was urine, 0.92 of the final specific immunoactivity could be attained.

Chromatography on DEAE-cellulose. (Fig. 1) Most of the immunoactivity was adsorbed with a DEAE-cellulose column and eluted as a broad large peak at conductivity ranging from 2 to 7 mmhos.

Gel chromatography on Sephadex G-100. (Fig. 2) Although two major peaks were observed by absorbance at 280 nm, the immunoactivity of the α -subunit was determined only in the preceding peak. This fraction containing high immunoactivity of α -subunit was collected, dialyzed against distilled water and lyophilized.

Fig. 1. Purification of α -subunit from undifferentiated carcinoma by chromatography on DEAE-cellulose. The lyophilized powder was dissolved in 50 ml of 0.005 M Tris-HCl buffer, pH 8.0 and applied to a DEAE-cellulose column (2 $\times$ 30 cm) equilibrated with the same buffer. The elution was performed by linear gradient of NaCl concentration, from 0-0.3 M, in 500 ml 0.005 M Tris-HCl, pH 8.0. Five ml fractions were collected with a flow rate of 15 ml/hour.

Fig. 2. Purification of α -subunit from undifferentiated carcinoma by gel chromatography on Sephadex G-100. The major peak from DEAE-cellulose chromatography was applied to a Sephadex G-100 column (2.8 $\times$ 96 cm) in 0.05 M sodium phosphate buffer, pH 7.4. Three ml fractions were collected with a flow rate of 10 ml/hour.

Table 1. Extraction and purification of α -subunit from urine of patient with undifferentiated carcinoma

	Total Protein (mg)	Total Alpha (mg)	Specific Activity (mg alpha/mg protein)	Recovery (%)
URINE	518	72	0.14	100
KAOLINE-ACETONE	123	34	0.28	47
DEAE-C	31	18	0.58	25
SEPHADEX G-100	12	11	0.92	15

Fig. 3. Partial purification of α -subunit from urine of normal pregnant women by gel chromatography on Sephadex G-100 in the same conditions as described for the purification of α -subunit from urine of the patient.

Fig. 3 shows the results of gel chromatography of the extracts from urine of normal pregnant women on Sephadex G-100. Although three major peaks were observed by absorbance at 280 nm, the immunoactivity was determined in both second and third peaks. The immunoactivity of the second peak was ascertained to be due to the cross-reactivity of large amounts of hCG. The fractions under the peak of hCG- α immunoactivity were collected, dialyzed against distilled water and lyophilized. The specific immunoactivity of this fraction was 0.26 (mg α -subunit/mg protein). This partially purified α -subunit was subjected to isoelectric focusing and a combination study.

Elution Profiles on Sephadex G-100

Fig. 4 depicts the elution profiles of ^{125}I - α -subunit from undifferentiated carcinoma, ^{125}I -hCG and ^{125}I -hCG- α on a Sephadex G-100 column. V_e/V_o of α -subunit, hCG and hCG- α were 1.48, 1.27 and 1.71, respectively.

Immunoactivity in hCG- α radioimmunoassay

Fig. 5 shows the dose response line of a purified α -subunit from undifferentiated carcinoma in the homologous hCG- α RIA. The α -subunit exhibited virtually identical immuno-antigenicity against hCG- α antiserum with

Fig. 4. Elution profiles of iodinated α -subunit from undifferentiated carcinoma, standard hCG and hCG- α on Sephadex G-100 column (1.5 $\times$ 90 cm). V_e/V_o of α -subunit = 1.48, hCG = 1.27, hCG- α = 1.71

Fig. 5. The dose response line of purified α -subunit from undifferentiated carcinoma in the homologous hCG- α RIA

that of standard hCG- α .

SDS disc electrophoresis

Fig. 6 shows the results obtained following SDS disc electrophoresis of a purified α -subunit from undifferentiated carcinoma and hCG (KOBE) after mercaptoethanol reduction. hCG was separated into two different migrating bands (Fig. 6, gel 2), whereas α -subunit from undifferentiated carcinoma appeared as a single band (Fig. 6, gel 1). Molecular weights estimated from their mobilities showed that the faster and slower moving bands of hCG were about 19,000 and 28,000

Fig. 6. SDS disc electrophoresis of α -subunit from undifferentiated carcinoma (gel 1), hCG (gel 2) and standard molecular markers (gel 3)

respectively, and that of the α -subunit was about 18,000 (Fig. 7).

Amino acid analysis

The results of amino acid compositions of an α -subunit from undifferentiated carcinoma and the average of various glycoprotein hormone α -subunits by literature analyses quoted from Weintraub *et al.* (1975) are listed in Table 2. There is considerable similarity in their amino acid compositions. The aspartic acid, serine, glutamic acid and glycine content in the α -subunit was slightly higher than the literature analyses of standard α -subunits, but the half-cystine and methionine content was lower conversely.

Isoelectric focusing

Fig. 8 depicts the isoelectric focusing pat-

Fig. 7. Molecular weight of α -subunit from undifferentiated carcinoma estimated by SDS disc electrophoresis. Mobilities were expressed as percentage of that of bromophenol blue used as reference.

Table 2. Amino acid analysis of α -subunit from undifferentiated carcinoma compared to literature analyses of various human α - α -subunits

AMINO ACID*	α -sub-unit**	human α -sub-units***
Lysine	6.2	6.0
Histidine	3.2	3.0
Arginine	3.6	3.6
Aspartic acid	7.5	6.7
Threonine	8.5	8.6
Serine	9.2	8.3
Glutamic acid	11.4	10.2
Proline	7.9	7.7
Glycine	6.7	5.4
Alanine	5.7	5.2
Half-cystine	6.9	9.4
Valine	7.6	7.5
Methionine	1.9	3.1
Isoleucine	1.7	2.1
Leucine	4.3	4.9
Tyrosine	3.2	4.0
Phenylalanine	4.5	4.3

* expressed as moles/mole α -subunit

** α -subunit from undifferentiated carcinoma

*** Average of various α -subunits quoted from Weintraub *et al.* (1975)

Tryptophan was not examined.

Fig. 8. The isoelectric focusing patterns of α -subunit from undifferentiated carcinoma (panel A), α -subunit from urine of normal pregnant women (panel B) and standard hCG- α (panel C)

terns of α -subunit from undifferentiated carcinoma (panel A), an α -subunit from urine of normal pregnant women (panel B) and standard hCG- α (panel C). The major components of tumor derived α -subunit and α -subunit from urine of normal pregnant women were distributed over the pH range from 3.5 to 6.0, whereas standard hCG- α was distributed in the fractions ranging from pH 6.0 to 8.0.

Fig. 9. Percent combination of α -subunit from undifferentiated carcinoma and standard hCG- α with hCG- β , as measured by hCG RIA

Combination of alpha- and beta- subunits (Fig. 9)

While the recombination between hCG- α and hCG- β , which were separated from hCG by urea *in vitro*, increased with the time course at 4°C and 27°C, the α -subunit from undifferentiated carcinoma showed scarcely any combining activity with hCG- β at either temperature. The assay value of the samples from the incubation tube of the α -subunits and hCG- β was 258 ± 16 ng/tube at each point of the incubation in hCG-RIA. This value practically corresponded to the crossreaction (0.7%) of the α -subunit plus hCG- β for hCG-RIA.

Discussion

An α -subunit could be extracted and purified from urine of the patient with undifferentiated carcinoma. This tumor was ascertained immunohistochemically to be an isolated α -subunit producing tumor, and both immunoreactive hCG and free hCG- β were undetectable in urine and serum of the patient.

Ectopic syntheses of subunits of glycoprotein hormones have been demonstrated in many nontrophoblastic tumors and tumor-

derived cell lines (Weintraub and Rosen, 1973; Tashjian *et al.*, 1973; Rosen and Weintraub, 1974; *et al.*, 1976). Weintraub *et al.* (1975) extracted and highly purified α -subunit (A.L.- α , 1.84 of specific activity) from gastric carcinoid tumor tissue, and analyzed its amino acid composition. From the present results of the amino acid analysis of α -subunit from undifferentiated carcinoma, there was considerable similarity between tumor derived α -subunit and standard α -subunit compositions from the literature analyses. However, Weintraub *et al.* (1975) reported that the amino acid composition of A.L.- α showed certain differences from standard, especially high glycine content. Benveniste *et al.* (1979) also pointed out the size heterogeneity of JEG- α (choriocarcinoma cell origin) and suggested the possibility that minor heterogeneity existed in its protein moiety. On the other hand, Ruddon *et al.* (1980) have demonstrated that subunits secreted by JAG cells (choriocarcinoma cell origin) are heterogenous and appear to be a mixture of glycoproteins with simple high-mannose and complex sialic acid-containing oligosaccharides. In the present study, since α -subunit from undifferentiated carcinoma exhibited similar properties to standard hCG- α in regard to the parallel dose response and dose inhibition in homologous hCG- α radioimmunoassay system, it was considered that little difference affecting the immunoactivity may exist between tumor derived α -subunit and standard hCG- α . This problem should be elucidated by the study of the amino acid sequence of the α -subunit.

It has been demonstrated that the various ectopically produced α -subunits have apparent larger molecular weights in gel exclusion chromatography. Also in the present study, the molecular weight of an α -subunit from an undifferentiated carcinoma determined by gel chromatography on Sephadex G-100 was larger than that of standard hCG- α . By SDS disc electrophoresis, however, the molecular weight of α -subunit from undifferentiated carcinoma was smaller than that of hCG- α

separated by mercaptoethanol reduction. Weintraub *et al.* (1975) have already reported such a discrepancy in molecular weight estimation of α -subunit (A.L.- α) between gel chromatography and by SDS disc electrophoresis, and suggested this is attributable to the possibility that the α -subunit might be binding more SDS than the standard α -subunit.

In the results of the isoelectroic fusing, the major components of α -subunit from undifferentiated carcinoma were observed in the fractions ranging from pH 3.5 to 6.0. This isoelectric focusing pattern was similar to that of partially purified α -subunit from the urine of normal pregnant women, but quite different from that of standard hCG- α which ranged from pH 6.0 to 8.0. Benveniste *et al.* (1979) showed that the great majority of JEG- α was dissolved in a peak pI of 4.8 which was virtually absent in standard hCG- α . The electric charge of glycoprotein was considered to be affected by various factors, such as the differences in amino acid and carbohydrate compositions and conformation. It is probable that the tumor derived from the α -subunit differs from standard hCG- α in regard to carbohydrate moiety, since the protein moiety of the α -subunit is considered to be virtually identical with that of standard hCG- α .

The results of the combination studies involving α - and β -subunits indicate that α -subunit from undifferentiated carcinoma does not actively combine with standard hCG- β . Although similar findings (Benveniste *et al.*, 1979; Weintraub *et al.*, 1977) have been shown in the various α -subunits, only A.L.- α showed a small but significant degree of combination in both RIA and RRA. By the preliminary study, the partially purified α -subunit from urine of normal pregnant women was not significantly active in combining with standard hCG- β (data not shown). Therefore, the nature of hCG- α obtained by the dissociation of hCG may be dissimilar to that of the urinary α -subunit, and freely secreted α -subunit might no longer have the ability to combine with hCG- β . We suppose that A.L.- α

has the ability to combine with the β -subunit because it originates from tumor tissue.

HCG may be synthesized *via* the association of α - and β -subunits which are synthesized from two different messenger RNAs (Boime *et al.*, 1978), and hCG- β may be a rate-limiting factor of hCG production in the placenta (Franchimont and Reuter, 1972; Chatterjee and Munro, 1977). Hence we speculate that the excess of α -subunit, which has not associated with β -subunit, may have undergone some intracellular modification of its chemical structure, and consequently, the freely secreted α -subunit electric charge changes and has no longer the activity to combine with the β -subunit.

We are now also studying the chemical properties and the combination of free α -subunit in placental tissue.

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