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INTRACELLULAR MOLECULAR SPECIES OF HUMAN CHORIONIC GONADOTROPIN FROM NORMAL BUT NON-CULTURED FIRST TRIMESTER PLACENTAL TISSUES

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Intracellular forms of human chorionic gonadotropin (hCG) were analyzed by SDS-polyacrylamide gel electrophoresis, protein blotting and immunological techniques in normal but non-cultured first trimester placentae.

Placental cells were found to contain the major components of the 23K and 19K forms of the beta-subunit and the 21K form of the alpha-subunit of hCG which remained sensitive to endoglycosidase H and Con A-Sepharose 4B and small amounts of mature (urinary) subunits. An unknown molecular species of the alpha-subunit (Mr=17K) that was not bound to Con A-Sepharose 4B was also detected. These intracellular molecular species accumulated in the placentae mainly during the first trimester.

These results suggest that hCG subunits accumulate in placental cells as predominant intermediates containing high-mannose oligosaccharides.

The urinary human chorionic gonadotoropin (hCG) was established as the secreted form of chorionic gonadotropin from the placental trophoblast. hCG consists of a noncovalently bound dimer of alpha- and beta-subunits. The urinary form of hCG was well characterized, i.e., its amino acid (1,2) and sugar sequences (3-6) were determined.

The newly synthesized alpha- and beta-subunits of hCG in placentae appear as precursor molecules (7) that process a series

The abbreviations used are : hCG, human chorionic gonadotropin ; anti-alpha, anti-alpha subunit antibody ; anti-beta, anti-beta subunit antibody ; SDS, sodium dodecyl sulphate ; PMSF, phenylmethylsulfonyl fluoride ; EDTA, ethylenediaminetetraacetic acid ; endo H, endoglycosidase H (EC.3.2.1.96)

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of co- and post-translational modifications before these subunits are secreted. Intracellular and secreted molecular species of hCG subunits have been studied by Ruddon et al. using cultured normal placental tissue and choriocarcinoma cells (8-12) using the technique for pulse and chase experiments. They suggested that alpha- and beta-subunits are pooled as high-mannose forms before secretion and the processing of asparagine-linked oligosaccharide units such as deglycosylation by mannosidase to produce core region and transglycosylation of oligosaccharides to the terminal region is the rate-limiting step for secretion. It is likely that the half-lives of these high-mannose forms are quite long ($\geq$1 hr). If so, one can expect to detect these species in the non-cultured, aborted placentae themselves. These intermediates are useful tools for studying the processing enzymes of hCG subunits.

In this study, high-mannose forms of hCG subunits were detected as major intracellular forms in normal, fresh but non-cultured first trimester placental tissues.

MATERIALS AND METHODS

Extraction of placental proteins:

First trimester (8-11 weeks' gestation) human placental chorionic tissues were obtained at the time of elective abortion. One gram of the chorionic tissue was dipped immediately in 1 ml of extraction buffer (40 mM Tris-HCl, pH 8.7, containing 2 % KCl, 1 mM PMSF and 1 mM EDTA) and homogenized with polytrone at 4°C. The suspension was centrifuged at 7,500 x g for 1 min. The supernatant, usually 5 to 7 mg proteins per ml, was recovered as the tissue extract and used for protein blotting analysis and endoglycosidase H digestion.

SDS-Polyacrylamide Gel Electrophoresis and protein blotting:

Tissue extract (5-10 µl of which contained 2-4 IU of hCG) was mixed with an equal volume of SDS-polyacrylamide gel electrophoresis sample buffer (150 mM Tris-HCl, pH 6.8, 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol and 0.001 % bromophenol blue), boiled for 5 min, cooled and centrifuged at 15,000 rpm (TOMY-15A) for 5 min. The supernatant was analyzed by SDS-polyacrylamide gel electrophoresis using an 11.5 % polyacrylamide gel which was prepared as described by Laemmli (13). At the end of electrophoresis, the proteins on the polyacrylamide gel were transferred to a Durapore (GVHP) filter (Millipore Ltd.) electrophoretically in 25mM Tris,192mM glycine and 20 % methanol as described by Towbin et al.(14). After blotting the proteins,

the filter was cut and used for immunological detection or staining with coomassie brilliant blue R-250. The residual polyacrylamide gel was also stained to check the efficiency of blotting (usually 90-95 % of proteins was transferred). The marker polypeptides used were bovine serum albumin (Mr=68K daltons), hen egg albumin (Mr=45K daltons), chymotrypsinogen (Mr=25K daltons) and cytochrome c (Mr=12.5K daltons).

Immunological detection of chorionic gonadotropin subunits:

Rabbit antisera against the alpha- and beta-subunits of hCG were generously supplied by Dr. Y. Yuki (The Japan Chemical Research). Cross-reactivities of anti-alpha-subunit antibody (anti-alpha) with beta-subunit and hCG were below 0.1 % and ca. 2%, respectively. Cross-reactivities of anti-beta-subunit antibody (anti-beta) with alpha-subunit, hCG and leutropin were below 0.1 %, ca. 5 % and ca. 0.25 %, respectively, as determined by radioimmunoassay. The electrophoretic blots were soaked in 3 % bovine serum albumin in TS (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl) for 1 hr at room temperature to saturate additional protein binding sites. After the blots were rinsed with TS, the proteins of placental tissue on the blots were incubated with shaking for 1 hr at room temperature with anti-alpha or anti-beta in the TS buffer containing 3 % bovine serum albumin. Antisera were usually diluted 200 times with the above buffer. The filters were washed with TS containing 0.1 % Triton X-100 and 0.1 % SDS (TSTS) and incubated with the second antibody in TSTS containing 3 % bovine serum albumin for 1 hr at room temperature with shaking. As the second antibody, anti-rabbit IgG [^{125}I]- labeled whole antibody from donkey (10μCi/μg, Amersham) was used at a concentration of 0.5μCi/ml. The filters were washed extensively with TSTS and dried. They were then exposed to Fuji new AIF RX film for 5 to 20 hours.

Digestion of Tissue Extracts with Endoglycosidase H:

Endoglycosidase H (endo-beta-N-acetyl-D-glucosaminase, endo H, EC.3.2.1.96.) was purchased from Seikagaku Kogyo Co. LTD.,Tokyo,Japan. The tissue extracts (600 μg of protein) were dialyzed against 0.2 M citrate-phosphate buffer, pH 5.0, in the presence of 1 mM PMSF and 1 mM EDTA. 0.01 unit of endo H was added to the 100 ul of dialyzate, and the solution was incubated at 37° C for 12 hrs.

Preparation of Urinary hCG and its Asialo Form:

Urinary hCG was purified from crude hCG (5,000 IU/mg, purchased from Mochida, Inc., Tokyo, Japan) by Con A Sepharose 4B, Sephadex G-100 and DEAE cellulose chromatographies as described in (15). The purified hCG had an activity of 12,000 IU/mg. Subunits of hCG were obtained by the method of Swaminathan and Bahl (16). The asialo urinary hCG was prepared by digestion with neuramidase (Sigma Chem. Co.) in 0.15 M acetate buffer, pH 4.5, containing 0.45 M NaCl and 0.027 M $CaCl_2$ at 37°C for 24 hrs.

Con A affinity chromatography:

Tissue extracts (350 μg of protein) were loaded on a Con A-Sepharose 4B column (Pharmacia P-L Biochemicals) and the chromatography was performed at room temperature by the method described by Kornfeld et al. (17). The non-adsorbed fraction and adsorbed fractions that were eluted with 10 mM and 500 mM methyl-alpha-D-glucoside, respectively, were analyzed by blotting and immunological assays.

Miscellaneous:

The hCG international unit (IU) was measured by using a Titration-Set made by Carton-Wallace Inc. (Cranbury, N.J.,U.S.A.). Protein was determined by the method of Bradford (18) using bovine serum albumin as a standard. All other chemicals used were standard commercial products.

RESULTS AND DISCUSSION

Intracellular molecular species of hCG in the first trimester placenta (10th week of pregnancy) were analyzed by anti-alpha and anti-beta, as shown in Fig. 1. The aborted placental chorionic tissue was immediately homogenized in the presence of protease inhibitors and the extracts were loaded on a polyacrylamide gel containing SDS, blotted on a Durapore filter and incubated with anti-alpha or anti-beta and then with [^{125}I]-labeled anti-rabbit IgG goat serum as the second antibody.

Two major and one minor protein bands were detected with anti-beta (Fig. 1, lane 2). Immunological interaction of these protein bands with anti-beta was blocked by the presence of excess beta-subunit from urinary hCG, but not alpha-subunit (data not shown). The molecular weight of the minor protein band was 31K, and it migrated the same as the beta-subunit of urinary hCG (mature form). The major bands have smaller molecular weights than the mature form, being 23K and 19K. That is, only a small amount of mature (urinary) beta-subunit was detected intracellularly in

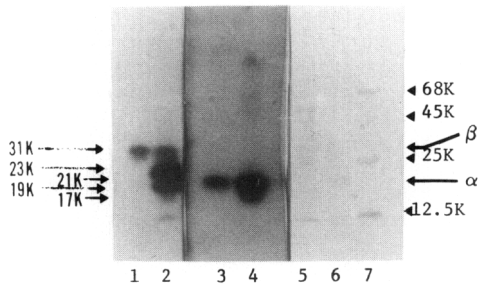

Fig. 1. Identification of intracellular forms of hCG subunits in first trimester placental tissues. Proteins of tissue extract from aborted first trimester placenta (10th week of pregnancy) were separated by SDS polyacrylamide gel electrophoresis and then analysed by anti-alpha or anti-beta after the proteins were blotted on a Durapore filter. Urinary hCG (0.16 μg for lanes 1, 3 and 1.9 μg for lane 6), tissue extract (30 μg for lanes 2, 4 and 5) and marker proteins (5μg of each proteins, lane 7) were blotted onto a Durapore filter as described under "MATERIALS AND METHODS". The filter was cut and incubated with anti-beta (lanes 1 and 2) or anti-alpha (lanes 3 and 4). Lane 5 to 7 were stained with coomassie blue. α, alpha-subunit; β, beta-subunit.

the normal and non-cultured first trimester placental chorionic cells. This suggests that the major intracellular forms of the beta-subunit existed as smaller molecular species, i.e. intermediates, rather than its mature form.

With anti-alpha, one major and one minor protein bands were detected (Fig. 1, lane 4). The major band (Mr≈21K) migrated almost the same as the urinary (mature) alpha-subunit and the other protein band, which showed weaker radioactivity, migrated significantly faster than the protein band corresponding to the mature (urinary) alpha-subunit and had a molecular weight of about 17K daltons. Immunological detection was blocked by the presence of excess alpha-subunit from urinary hCG, but not by beta-subunit (data not shown). These facts indicate that intracellular forms of the alpha-subunit might have existed in forms very similar to the mature form, or as the mature form itself as well as molecular species which were smaller than the mature subunit.

These results can not be due to an intracellular protease activity that might be producing the lower molecular weight species of the hCG subunits for the following reasons; 1) addition of the protease inhibitors during homogenization and preparations, and 2) these molecular species have been detected in all preparations of so-called first trimester placentae (8-11 weeks of pregnancy, Fig. 2, A and B).

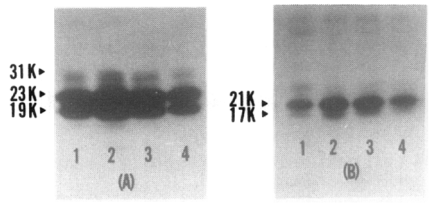

Fig. 2. Identification of intracellular forms of hCG subunits in various preparations from first trimester placentae. Proteins of tissue extract (30 μg) from first trimester placentae at various weeks of pregnancy were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by anti-beta (A) or anti-alpha (B) using the same conditions as described in the legend of Fig. 1. Lanes 1 to 4 were 8th, 9th, 10th and 11th week of pregnancy.

Using cultured choriocarcinoma cells (JAR) and normal first trimester tissues for pulse and chase studies, Ruddon et al. reported that JAR and cultured first trimester tissue synthesize intracellularly Mr=24K and 18K forms of the beta-subunit and Mr=18K and 15K forms of the alpha-subunit of hCG which are differnt and smaller molecular species than mature (urinary) hCG subunits (8-12). It is likely that our Mr=23K and 19K forms of the beta-subunit and Mr=21K and 17K of the alpha-subunit correspond to the Mr=24K and 18K forms of the beta-subunit and Mr=18K and 15K forms of the alpha-subunit,respectively.

They also mentioned that these intracellular forms have a long half-life in the cell ($\geq$1 hr) (11). This probably accounts for why we were able to detect similar intracellular intermediate forms of hCG subunits in the normal but non-cultured first trimester placentae.

Two asparagine-linked oligosaccharide chains have been determined (3, 5) on the alpha- and beta-subunits of hCG. In addition, the beta-subunit has four serine-linked oligosaccharide chains (4). It is well known that the intracellular glycosylated precursors of glycoproteins contain high-mannose asparagine-linked oligosaccharide chains which are subsequently processed and added to complex oligosaccharides (19, 20). Ruddon et al. have also reported that their intracellular forms remain sensitive to endo H (11), an enzyme that cleaves off the high-mannose oligosaccharide from glycoproteins specifically, but does not attack the mature forms of these glycoproteins containing complex oligosaccharides.

We have investigated whether these intracellular molecules of hCG subunits from normal but non-cultured first trimester placentae have this sensitivity to endo H. The tissue extracts were dialyzed against endo H digestion buffer and incubated with endo H as described in "MATERIALS AND METHODS". After digestion

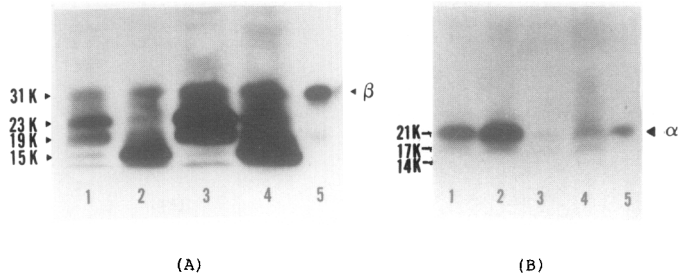

Fig. 3. Endo H sensitivity of intracellular forms of hCG subunits. The tissue extract (10th week of pregnancy) was digested with endo H as described under "MATERIALS AND METHODS" and an aliquot was subjected to SDS-polyacrylamide gel electrophoresis and analyzed with anti-beta (A) or anti-alpha (B) using the same conditions as described in the legend of Fig. 1. (A): lanes 1 and 3, 0 time of endo H digestion (70 μg and 140 μg of proteins for lanes 1 and 3, respectively); lanes 2 and 4, 12 hr digestion of the same proteins as lanes 1 and 3,respectively; lane 5, urinary hCG (1.9 μg). (B): lanes 1 and 2, 0 time of endo H digestion (70 μg and 140 μg of proteins for lanes 1 and 2, respectively); lanes 3 and 4, 12 hrs digestion of the same proteins as lanes 1 and 2, respectively; lane 5, urinary hCG; α, alpha-subunit; β, beta-subunit.

with endo H, a new protein band (Mr=15K) appeared instead of the original 23K and 19K protein bands of the intracellular beta-subunit, however, the 31K (mature) form was not attacked by endo H, judging from the intensity of the band on the autoradiogram (Fig. 3A). This result suggests that both intracellular forms of the beta-subunit were sensitive to endo H, but the mature form was not. Furthermore, all intracellular forms of the beta-subunit were adsorbed to a Con A column (Fig. 4A) and these protein bands did not correspond to the asialo form of the mature beta-subunit (data not shown). These results indicate that the 23K and 19K forms of the beta-subunit were high-mannose forms and the 31K form was a mature form, as reported by Ruddon et al. (8, 11).

On the other hand, both original intracellular forms of the alpha-subunit were detected after endo H digestion as well as a new protein band (Mr=14K) (Fig. 3B). The 21K form has affinity to Con A-Sepharose 4B but the 17K form does not (Fig. 4B). In addition the intensity of the 17K protein band before and after

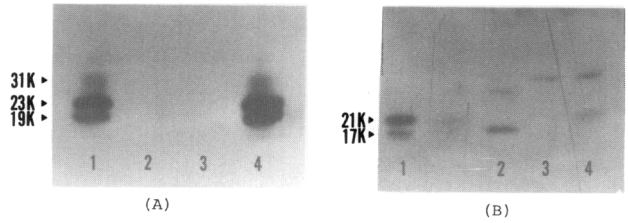

Fig. 4. Affinity of intracellular forms of hCG subunits to Con A-Sepharose 4B. Tissue extract was dialyzed against Tris-buffered saline containing 0.15 M NaCl, 0.01 M Tris, pH 8.0, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ and loaded on a column (1.5 ml) of Con A-Sepharose 4B. Proteins were eluted with the above buffer containing 10 mM (lane 3) and 500 mM (lane 4) of methyl-alpha-D-glucoside. After concentration of each fraction by ultrafiltration (Molcut II, Millipore Co.), aliquots were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by anti-beta (A) or anti-alpha (B) under the same conditions as described in the legend of Fig. 1. Lane 1, loaded sample; lane 2, non-adsorbed fraction.

endo H digestion was almost the same. These results suggest that only the intracellular 21K form, and not the 17K form, of the alpha-subunit is susceptible to endo H. The molecular species of the 17K of the alpha-subunit has not yet been determined. Following endo H digestion, the lower recovery of radioactivity of the newly appeared 14K form compared with the 21K form before digestion may suggest that; 1) the 21K form is a mixture of mature form which is resistant to endo H digestion and a high-mannose form of alpha-subunit, because sufficient digestion with endo H failed to diminish the 21K protein band (residual 21K form may be the mature form) and 2) an intracellular form of the alpha-subunit cleaved off the high-mannose region is very susceptible to an endogenous protease in spite of the presence of protease inhibitors in the digestion buffer.

Ruddon et al. have shown the clear kinetics of endo H digestion(11). The recoveries from digested bands after endo H treatment seem to be reasonable, contrary to our results, because they have digested the immunoprecipitated intracellular forms which are supposed to be nearly endogenous protease free.

In conclusion, intracellular precursors of hCG subunits containing high-mannose oligosaccharides, as well as unknown molecular species in the case of the alpha-subunit, were detected in normal aborted first trimester placental chorionic cells. These precursors must be trimmed by endogenous glycosidases and modified by addition of terminal sialic acid-containing complex oligosaccharides by a glycosyltransferase in the cells before or at the time of secretion.

It is interesting to note and feasible to study these processing enzymes for the synthesis and secretion of hCG subunits, i.e. intracellular sites of deglycosylation and glycosylation, because these precursors could be purified from aborted placentae as substrates.

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