Chorionic Gonadotropin Synthesized in Cultivated Trophoblast*

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Synopsis

Using 3H-proline as a marker of synthesized hCG, human chorionic tissue of the first trimester of pregnancy was cultivated in a synthetic medium which contained no serum component. By a combination of Sephadex G-100 gel filtration and DEAE-cellulose chromatography, biosynthesized 3H-labeled hCG in the medium was purified. On DEAE-cellulose chromatography immunoreactive hCG was eluted at a conductivity of 4.2-6.5 and 7.8-11.3 mmoles, whereas native hCG had been eluted only at a conductivity of 1.5-4.2 mmoles. On polyacrylamide disc gel electrophoresis 3H-radioactivity and immunoreactivity of hCG showed a peak in the same segment of the disc which corresponded to the migration area of native hCG. Sialic acid content of the biosynthesized 3H-labeled hCG was 0.04-0.05% whereas it was 3.0-3.8% in native hCG. In Ouchterlony immunodiffusion the biosynthesized hCG fraction eluted at a lower conductivity of 4.2-6.5 mmoles on DEAE-cellulose clearly made a precipitation line only with anti-hCG serum. On the other hand the fraction eluted at a higher conductivity of 7.8-11.3 mmoles made precipitation lines against both anti-hCG and anti-hCFSH serum. These results certified that hCG labeled with 3H-proline was biosynthesized actively in our culture system and it might be suspected that the biosynthesized hCG had some different properties from native hCG, and that hCFSH could be biosynthesized in vitro, too.

It was demonstrated that human chorionic gonadotropin (hCG) with high LH-like activity and human chorionic FSH (hCFSH) with high FSH-like activity were isolated separately from lyophilized powder of trophoblastic tissue and that the ratio of these two hormones varied at the different stages of pregnancy (Ashitaka, 1970; Tojo et al., 1972, 1973). These findings facilitated us to investigate the biosynthesis of hCG in placenta.

Up to now there have been little informations available on the biosynthesis of hCG except reports of Benagiano et al. (1972) and Patrito et al. (1973). This report describes the biosynthesis of hCG in vitro in a culture system and properties of biosynthesized hCG comparing with those of native hCG isolated from lyophilized chorionic tissue.

Materials and Methods

Preliminary study

Human chorionic tissue obtained aseptically from the therapeutic abortion in the first trimester of pregnancy was minced into fragments of approximately 1 mm³ and was cultivated by a modification method of Trowell (1959) in a synthetic medium “199” containing 100 IU of penicillin and 100 µg of streptomycin per ml. No serum components were added to the medium.
A filter paper, 25 mm in diameter, was placed in a glass petri-dish (70 mm in diameter), onto which 50 mg of the minced fragments were transferred. Fifteen milliliters of the culture medium was pipetted into one dish. The dish was then placed in CO₂-incubator (type B, Ikemoto Rika Kogyo, Tokyo), gassed with 5% CO₂-95% air and incubated at 37°C. Using ³H-thymidine (5.0 Ci/mmol, Amersham), ³H-uridine (5.0 Ci/mmol, Amersham), or ³H-proline (653 mCi/mmol, Amersham), viability of the cultivated trophoblast was checked with radioautographic techniques with the dipping method (Kopriwa and Leblond, 1962). HCG activity in the chorionic tissue was estimated immunologically by hCG radioimmunoassay (Tojo et al. 1969). The ratio of the amount of hCG to the total protein amount in the cultivated tissue which was measured by the method of Kjeldahl (Ballentine, 1957), and the ratio to total protein amount in the medium by the method of Lowry et al. (1951) or the ultraviolet absorption at 280 mµ were calculated, respectively.

**Extraction and purification of biosynthesized hCG**

Cultivation of the chorionic tissue in the first trimester of pregnancy was carried out in ten dishes for 5 days which contained radioactive medium prepared by adding 5 µCi of ³H-proline per ml. After the cultivations were terminated extraction and purification of hCG from the medium were performed. The concentrated medium was charged into a Sephadex G-100 column (2 x 90 cm), equilibrated in the same condition and was eluted by a linear gradient elution at 0.4M Tris-HCl, pH 8.6. The effluent fractions were read at 280 mµ by the ultraviolet absorption. ³H-radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrophotometer and hCG activity was measured by radioimmunoassay.

**Biochemical and immunological analysis of synthesized hCG**

Fractions on DEAE-cellulose which had hCG activity by radioimmunoassay were submitted to polyacrylamide disc gel electrophoresis by the technique of Ornstein (1964) and Davis (1964), using three columns for one fraction. One of the columns was stained with amidoblack 10-B to detect the protein. The other two were sliced transversely into segments of a thickness of 2.4 mm with a device of Chrambach. Each segment was assayed for ³H-radioactivity and immunoreactivity of hCG.

The immunological cross-reactivity of radioactive hCG fraction of DEAE-cellulose was estimated by a modified method of Ouchterlony (1953) using specific anti-hCG serum (Ashitaka et al., 1970a) and anti-hCFSH serum (Ashitaka et al., 1972). The content of sialic acid of radioactive hCG fraction on DEAE-cellulose was examined by the thiobarbiturate method of Warren (1959).

**Results**

**Viability of the cultivated trophoblast**

Chorionic tissues cultivated for 3 days

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*Fig. 1. Radioautographs of the chorionic tissue cultivated for 3 days.*

The silver grains of ³H-thymidine (a), ³H-uridine (b) or ³H-proline (c) were mostly found in cytotrophoblast and stromal cell but little in syncytiotrophoblast after two hrs exposure to each of the isotopes (×140).
were exposed to \(^3\text{H}\)-thymidine, \(^3\text{H}\)-uridine, or \(^3\text{H}\)-proline for 2 hrs. Radioautographic silver grains were strongly recognized in cytotrophoblasts and stromal cells, while little was recognized in syncytiotrophoblasts (Fig. 1a-c). In case of the tissue cultivated for 7 days, however, \(^3\text{H}\)-thymidine uptake was admitted only a little in cytotrophoblasts and stromal cells, and \(^3\text{H}\)-proline incorporation was not recognized whereas \(^3\text{H}\)-uridine uptake still remained strongly.

**Productivity of hCG**

Protein contents of the cultivated tissue increased transiently from 4 to 16 hrs of culture, while it decreased gradually, thereafter (Tojo et al., 1972). The amount of hCG in the cultivated tissue was 52.1 µg on the first, 33.6 µg on the second and 30.6 µg on the third day of culture. The ratio of the amount of hCG to the total protein in the tissue was 0.46% on the first, 0.28% on the second and 0.30% on the third day of culture. The ratio in the culture medium increased subsequently throughout the cultivation; 3.67% on the first, 21.38% on the second and 59.76% on the third day of culture. The ratio in the whole system of the culture varied from 0.64% on the first to 1.07% on the third day of culture (Fig. 2).

**Extraction and purification of the biosynthesized hCG**

Pooled medium from 10 dishes cultivated for 5 days was concentrated into about 5 ml in a rotary vacuum evaporator. The concentrated medium was gel filtrated on a Sephadex G-100 column and was separated into two fractions: tube 10–19 and 29–43. In the former fraction protein concentration was closely coincided with \(^3\text{H}\)-radioactivity and immunoreactivity of hCG. On the other hand, the latter fraction corresponded to the elution area of free amino acids and salts (Fig. 3). Moreover, by calculating the specific \(^3\text{H}\)-radioactivity and hCG-immunoreactivity per mg of protein of each effluent volume, it was observed that there was a mutual relationship between the two activities in the former fraction. The former fraction was further purified on DEAE-cellulose column chromatography. The adsorbed fraction of DEAE-cellulose was eluted at a conductivity of 2.5–6.0 mmhos and 7.0–11.0 mmhos. Immunoreactivity of hCG was admitted in the fractions at a conductivity of 4.2–6.5 and 7.8–11.3 mmhos (Fig. 4).

**Biochemical and immunological properties of biosynthesized hCG**

One fraction eluted at a low conductivity
Fig. 3. Gel filtration of the concentrated culture medium on Sephadex G-100.
The culture medium was separated into two fractions. In the former fraction (tube #10–19), all of protein concentration, ³H-radioactivity and immunoreactivity of hCG coincided closely. The latter fraction (tube #29–43) corresponded to the elution area of free amino acid and salts.

Fig. 4. Chromatography of the radioactive protein on DEAE-cellulose.
The adsorbed protein was eluted by linear gradient elution method. Protein concentration of the effluent fraction formed two peaks with a similar distribution of ³H-radioactivity. Immunoreactivity of hCG was also admitted in two fractions: conductivity of 4.2–6.5 mmhos and 7.8–11.3 mmhos.
Fig. 5. Electrophoretic patterns of the radioactive hCG fractions on DEAE-cellulose.

7% polyacrylamide disc gelectrophoresis was carried out with purified hCG (1), purified hCFSH (2) and radioactive hCG fractions on DEAE-cellulose at a conductivity of 4.2–6.5 mmhos (3) and 7.8–11.3 mmhos (4). The fraction eluted at a low conductivity had a few bands corresponding to the migration area of hCG. The other fraction eluted at a high conductivity had a dark band corresponding to the migration area of hCFSH and a few faint bands with low migration.

(4.2–6.5 mmhos) showed several bands with low migration on 7% polyacrylamide disc gel. 3H-radioactivity and hCG immunoreactivity on this disc showed a coincidental sharp peak at the segment #7. The other fraction with high conductivity (7.8–11.3 mmhos) showed a darker band with high migration and several faint bands with low migration which corresponded to the position of native hCG. 3H-radioactivity and hCG immunoreactivity at the position of low migration coincided completely, too (Fig. 5 and 6).

Sialic acid content of radioactive hCG fraction on DEAE-cellulose varied from 0.04% at a conductivity of 2.5–4.0 mmhos to 0.05% at a conductivity of 7.8–11.3 mmhos, while that of native hCG extracted from placental tissue had been 3.0–3.8% (Ashitaka, 1970).

In Ouchterlony immunodiffusion neither anti-hCG nor anti-hCFSH reacted with a sample eluted at a conductivity of 2.5–4.0 mmhos on DEAE-cellulose column. However, fractions eluted at a conductivity of 4.2–6.5 mmhos made a precipitation line only with anti-hCG serum. Fraction eluted at a conductivity of 7.8–11.3 mmhos made precipitation lines against anti-hCG and anti-hCFSH serum (Fig. 7).

Discussion

This investigation was undertaken in order to find out the hormonogenesis of hCG in chorionic tissue and the biochemical properties of synthesized hCG, utilizing a culture system. In our culture system no serum components were added for simplifying the procedures of purification and biochemical analysis of the biosynthesized hCG.

The radioautographic study showed that uptakes of 3H-thymidine and 3H-uridine were still recognized in the cultivated chorionic tissue of the 7th day of culture, suggesting that the tissue was viable even on that day. On the 3rd day of culture not only uptakes of 3H-thymidine and 3H-uridine but also that of 3H-proline was dominantly recognized in the tissue. These results indicated that the cultivated tissue could keep its viability and carried on the synthesis of proteins.

The ratio of the total amount of immunoreactive hCG to the total protein in the culture medium increased subsequently after the initiation of culture; 3.67% on the first and 59.76% on the third day. The ratio of the amount of immunoreactive hCG to that of total protein in tissue was varied from 0.46% on the first to 0.30% on the third day of culture. These results suggested that the cultivated chorionic tissue had an active function of hCG production in a serum free culture system and
In the present study, $^3$H-proline was used as a marker of biosynthesized hCG because of the high concentration of proline in hCG molecule (Ashitaka et al., 1970a). By a combination of Sephadex G-100 gel filtration and DEAE-cellulose chromatography, the radioactive fractions with hCG immunoreactivity were obtained from 5th day medium of culture. On polyacrylamide disc gel electrophoresis of radioactive hCG fractions, a single sharp peak of $^3$H-radioactivity was completely coincident with immunoreactivity of hCG. Migration of native hCG was also the same as that of the peak. These results confirmed the view that labeled hCG with $^3$H-proline was synthesized in this culture system.

Immunoreactivity of the biosynthesized hCG was found at a conductivity of 4.2–6.5 mmhos and 7.8–11.3 mmhos separately, while native hCG was eluted only at a conductivity of 1.5–4.2 mmhos, suggesting that the biosynthesized hCG was more tightly bound to anion exchanger and required salts of higher concentration to displace itself from a column.
at a given pH than did native hCG. This might be because no sialic acid was contained as a component in this culture medium. These results suggested that the biosynthesized hCG had some different structures or conformational changes in comparison with native hCG.

In Ouchterlony immunodiffusion, one biosynthesized hCG eluted at a conductivity of 4.2–6.5 mmhos made a precipitation line only with anti-hCG serum, while the other fraction eluted at a conductivity of 7.8–11.3 mmhos showed a faint precipitation line with anti-hCG serum and a clear precipitation line with anti-hCFSH serum. This fact indicated a possibility that hCFSH might be biosynthesized in our culture system, too. Benagiano et al. (1972) reported that using 14C-leucine hCG was synthesized, but FSH activity was not detected in any radioactive fractions obtained on DEAE-cellulose chromatography by radioimmunoassay for pituitary FSH. It was, however, reported that hCFSH did not cross-react with anti-FSH serum of pituitary origin in Ouchterlony immunodiffusion (Ashitaka et al., 1970b). FSH activity in the cultured medium should be detected by anti-hCFSH serum. This study is now being done and will be reported in the near future.

References