Oxytocin-like Substance in Human Placenta: Search for Uterine Contractile Substance

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Abstract. Placenta as a physiological source for oxytocic factor was implicated in our previous report, where we investigated the serum concentration of oxytocin (OT) and neurophysins during pregnancy, labor and lactation. The existence of immunoreactive (ir)-OT in placenta was suggested first by our study of the serum concentration in the umbilical vein, artery, and maternal circulation. The concentration of ir-OT in the placenta in the second trimester (20.8 ng/g-tissue) was 12-fold of that in the first trimester (1.6 ng/g-tissue). An immunohistochemical study on term placenta with the antiserum to OT revealed that ir-OT was mainly localized in the layer of syncytiotrophoblast. In vitro culture of trophoblast indicated that cycloheximide and prostaglandins (PGs) modulated the synthesis and/or release of ir-OT in the placenta. Further, the placental extract showed signs of mimicry of synthetic OT both in the elution profile through a carboxymethyl cellulose column and in bioactivity assayed by isometric tension of uterine muscle. The bioactivity and immunoreactivity of the extract were compared in the fractionated eluent of the extract on a Sephadex G-25 column. The results indicated that the bioactivity could be parallel with the immunoreactivity assayed by our RIA system, which was consistent with the fact that our antiserum neutralized the bioactivity of OT. We recently obtained a cDNA clone by immunoscreening from a human placental library, and the clone size was approximately 900 nucleotides. The clone was transfected into a Chinese hamster ovary cell line, the cultured media of which contained the recombinant ir-OT proved by assays of both immunoreactivity and bioactivity. A search for the major contractile factor in the initiation of labor is in progress.

Key words: Human placenta, Oxytocin, Rat uterus, Molecular cloning, Oxytocic agent, Labor onset

THE INITIATION of labor still involves questions to be answered, even though there has been much progress in reproductive technology and many new findings in reproductive science during this decade. When we investigated changes in the serum concentration of oxytocin (OT) and neurophysins during pregnancy, labor and lactation [1], we noticed different concentrations of OT in the umbilical vein, artery, and maternal circulation. This finding implied the existence of immunoreactive (ir)-OT in the placenta and its physiological role in labor. Mainly utilizing polyclonal antiserum generated in our laboratory, many efforts were devoted to analyzing its characteristics: molecular weight, the concentration change during pregnancy, and the localization in placenta along with its biological activity. At present, we are employing two methods to determine the substance: one is to determine the peptide sequence of the purified placental extract by high performance liquid chromatography (HPLC), another to clone cDNA from a human placenta library. In this paper, we will present our data and discuss a possible role of the placenta in delivery.
Materials and Methods

Hormone, antiserum, and radioimmunoassay

Anti-OT-antiserum was generated in New Zealand white female rabbits immunized with synthetic OT (Teikoku Hormone Mfg. Co., Ltd., Japan) conjugated to bovine serum albumin (BSA). Its specificity and potency were high enough for carrying out the following studies, and were reported previously by us [2]. The radioimmunoassay (RIA) of OT has been done consistently by a double antibody method with variations in inta- and inter-assays of less than 10%.

Preparation of placental extract

Placenta tissues of each trimester of pregnancy were minced and rinsed in 0.9 % NaCl solution, and homogenized with 5 volumes of 0.04 N acetic acid. After centrifugation (900 g, 30 min, 4 °C), the pH of the supernatants obtained was adjusted to 5.5 with 0.1 N NaOH. After 24 h on ice, the samples were centrifuged again and the supernatant was radioimmunooasayed for its ir-OT content.

Histological study of ir-OT in placenta

Immunohistological distribution in placenta was investigated with the antiserum and preimmune serum as a control. A modified peroxidase-anti-peroxidase-complex (PAP) method was carried out for immunohistochemical staining as reported previously [3] and was briefly as follows: The freshly obtained placenta was rinsed in 0.9 % NaCl solution and cut into 1.0 cm³ cubes, which were then fixed by means of 0.4 % glutaraldehyde, 4 % paraformaldehyde, 0.2 % picric acid in 50 mM phosphate buffer solution (pH 7.4) for 24 h. Frozen 16 μm slices cut with a cryostat were stained by a modified PAP method with 0.05 % diaminobenzidine and 0.005 % hydrogen peroxide. To confirm its histological localization, an adjacent slice of tissue was stained with hematoxyline and eosine (HE).

Tissue culture of trophoblast with drugs

After the histological study, we investigated the effects of prostaglandins (PGs) and a protein synthesis inhibitor on ir-OT content in cultured trophoblastic tissue. Trophoblastic tissues were prepared from term placenta obtained by cesarean section done before the onset of labor. A tissue block was aliquotted into 4 flasks (200 mg wet weight/flask) and cultured at 37 °C under 5 % CO₂ - 95 % air in 2 ml of Earle's medium 199 (M199, GIBCO) containing 25 mM HEPes, 15 % fetal calf serum, penicillin and streptomycin. After 2 h of culture, the tissues were washed twice with 1 ml of fresh incubation medium. The main incubation was continued for 4 to 24 h at 37 °C under 5 % CO₂ - 95 % air in 2 ml of the incubation medium containing several concentrations of cycloheximide, PG-E₂, and PG-F₂α. At the end of the incubation, the medium from each flask was transferred to a glass tube, and then a tissue extract was prepared according to the procedure described above. Samples were stored at −20 °C until assayed for ir-OT content by RIA.

Analysis by iron-exchange column chromatography

Placental extract and synthetic OT were analyzed by iron-exchange chromatography. The samples were eluted on a carboxymethyl cellulose (CMC) column with ammonium acetate buffer (pH 4.6) as eluent with its linear gradient concentration change of 0.002–0.4 M. Fractionated samples were assayed by RIA for OT.

Biological assay with Magnus apparatus

The oxytocic action of the investigated material was assayed with Magnus apparatus according to the method described by Perry WLM et al. [4]. The uteri of estrogen-primed rats were prepared for recording isotonic tension in a 5 ml bath filled with modified Ringer-Locke solution (25 °C).

Analysis of the extracts fractionated by gel-filtration chromatography

To compare the bioactivity and immunoreactivity of the extract, the following fine procedure for extraction was employed: After the first step of extraction with acetic acid described above, the solution was boiled for 30 min, then cooled and filtered through a Hyflo Supercel-layer. The solution obtained was concentrated to 1/5 volume in a vacuum-evaporator at 30 °C and lyophilized. Five
volumes (5 ml/g or ml) of glacial acetic acid was then added to the lyophilized powder. The acetic acid extraction was repeated 4 times, and the extraction was precipitated by the addition of 2.5 volumes of ethyl ether and 5 volumes of petroleum ether. The precipitate was washed with ethyl ether and dried in a vacuum. The resultant powder was applied to a Sephadex G-25 column with 0.1 M acetic acid. The amount of amino acid in the eluted solution was measured by the Folin-Lowry method. The fractions collected were lyophilized and assayed for their bioactivity and immunoreactivity by the methods described above.

Molecular cloning of ir-OT

A human placental cDNA library (Clontech, Palo Alto, CA, USA) was screened for ir-OT. The library, constructed from expression vector λ gt11, was infected to E. coli Y1089 with the phage. After plaque formation in Luria-Bertani broth (1 % agar), the fusion proteins expressed on nitrocellulose filters were screened with antiserum to OT and [125I]protein A in autoradiography. After repeated immunoscreening, the clone cDNA obtained was digested with EcoRI and electrophoresed on 1 % agarose gel. Parallel with this electrophoresis, northern blot hybridization analysis was also done with the cDNA obtained as a probe.

The PMhOT vector was constructed by ligating the cDNA obtained to a mammalian cell expression vector, pMANNeo. This vector was transfected into Chinese hamster ovary cells (CHO-K1), which were screened in a medium containing geneticine G418 (Gibco). Recombinant substance was produced in a serum-free culture medium (GIT) with dexamethasone (10⁻⁵ M). Immunoreactivity and bioactivity of the substance collected were assayed by RIA and Magnus apparatus respectively, which procedures have already been described.

Results and Discussion

Placental ir-OT contents in each trimester of pregnancy

The displacement curve for the placental extract in the RIA was parallel with that of synthetic OT (data not shown). The concentration of ir-OT in placenta of the second trimester (20.8 ng / g-tissue) showed a 12 fold-increase compared with that of the first trimester (1.6 ng/ g-tissue). Though the concentrations in the second and third trimester were approximately the same, the total content increased along with the gestational weeks solely dependent on placental weight: 12.7×10⁻³ (± 5.61), 4.10 (± 0.41), and 12.5 (± 1.22) mg/placenta (± SEM) in first (n=6), second (n=7), and third trimester (n=13), respectively. These data indicate that the human placenta in late gestation contains 4-5 times more OT than in the human neurohypophysis.

Localization of ir-OT in placenta and in vitro study of cultured trophoblast

Immunohistochemical staining of term placenta revealed that ir-OT is mainly localized in the outer layer of villous tissue. Compared with the adjacent HE stain specimen, the immunoreactive area is the syncytiotrophoblastic layer. The cytrophoblastic layer, interstitial area and vessels did not show any more staining than the background. The histologically identified localization does not necessarily indicate its physiological significance. We therefore tried to culture the trophoblastic tissue. Table 1(A) shows the effect of cycloheximide on ir-OT content in cultured trophoblastic tissue after 24 h. The protein synthesis inhibitor seems to block the synthesis of ir-OT. Further study with PGs suggested a physiological role of ir-OT in trophoblast (Table 1(B) and (C)). PG-E₂ increased ir-OT mainly of the tissue content in a dose dependent manner, while PG-F₂α dose-dependently increased ir-OT in both the tissue and the medium. It has been suggested that placental corticotropin releasing factor (CRF) might act as an important modulator in the inotropic effect of oxytocin on the myometrium [5]. In addition, our present data for PGs and ir-OT suggest that the placental ir-OT plays a pivotal role during the labor instead of the OT of pituitary origin, working synergistically with another modulator of placental origin.

Ion-exchange chromatography and bioactivity of ir-OT in placenta

The placental extract had similar elution profile in CMC column chromatography to that of synthetic OT (Fig. 1-(a)). The extract had as potent biological activity as synthetic OT which is illustrated in Fig. 1-(b-1), where both synthetic OT (A)
and placental extract (B) had bioactivity. This oxy-
tocic action of the extract was blocked by
incubation with the antiserum to OT for 2 days in
4 °C (Fig. 1-(b-1);C). Specific blockade by the anti-
serum was ascertained by the series of procedures
shown in Fig. 1-(b-2), where the bioactivity of the
extract (A) was abolished by the antiserum (B),
but not by normal rabbit serum (NRS; C); but, on
the other hand, neither NRS (D) nor the antiserum
(E) had any contractile activity. The specificity of
the antiserum is also shown in Fig. 1-(b-3). Sev-
eral peptide hormones had already been reported to
exist in human placental tissue: corticotropin-re-
leasing factor (CRF), growth hormone-RF, thyrotropin-RF and luteinizing hormone-RF [6-10].
Pearse [11] postulated that all tissues of neural crest
origin, including the placenta, contain such pep-
tides as RFs in the neurally-programmed region.
In this context, it seems natural that the ir-OT ex-
ists in human placental tissue and might play a
physiological role.

### Bioactivity and immunoreactivity of placental
extracts fractionated by gel-filtration
chromatography

To analyze both the bioactivity and immunore-
activity of the extract, the fine procedure described
in Materials and Methods was employed for the
extraction. The solution eluted on a Sephadex G-
25 column was divided into 7 fractions according
to the Folin-Lowry method (Fig. 2-(a)). Ir-OT in
each fraction was 56.9, 24.1, 4.6, 14.3, 2.6, 0.13, and
0.094 ng from the first through the seventh frac-
tion. The biological activity of each fraction was
assayed by the isometric tension of uterine muscle
(Fig. 2-(b)). The first, second and fourth fractions
had contractile bioactivity. This indicates that the
bioactivity could be parallel with the immunoreac-
tivity found by our RIA system, which was already
indicated by the fact that our antiserum neutral-
ized the bioactivity of OT.

### Molecular cloned substance and its bioactivity

The clone obtained by immunoscreening of a
human placental cDNA library was approximate-
ly 900 nucleotides in size (Fig. 3) as revealed by
both electrophoresis and Northern blotting [12].
The cloned cDNA was transfected into a CHO cell
line, the cultured media of which contained ir-OT
(approximately 200 pg/ml) as determined by RIA.
Furthermore, the bioactivity of this recombinant
ir-OT (r-OT) is demonstrated in Fig. 4. The r-OT had
a contractile effect on uterine muscle (Fig. 4-(a)),
which is proved again by negative control of an-
other recombinant material from CHO (E). This
potent biological effect of r-OT was neutralized by
the antiserum to OT as with synthetic OT (Fig.4-
(b)), but the nucleotide and amino acid sequences
responsible for the biological activity remain to be
clarified.

### Studies in progress

At present we are employing two methods to
determine the peptide sequences responsible for

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**Table 1. Effects of cycloheximide (A), prostaglandin-E$_2$
(B), and -F$_{2a}$ (C) on immunoreactive-oxytocin
(ir-OT) in cultured trophoblastic tissue**

<table>
<thead>
<tr>
<th></th>
<th>Incubation Time (h)</th>
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<tbody>
<tr>
<td>A) Cycloheximide (µg/ml)</td>
<td></td>
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<tr>
<td>0</td>
<td>1.80± 0.2</td>
</tr>
<tr>
<td>1</td>
<td>3.00± 0.35</td>
</tr>
<tr>
<td>10</td>
<td>1.85± 0.15</td>
</tr>
<tr>
<td>B) PGE$_2$ (µg/ml)</td>
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</tr>
<tr>
<td>0</td>
<td>0.47± 0.03</td>
</tr>
<tr>
<td>1</td>
<td>0.71± 0.10</td>
</tr>
<tr>
<td>10</td>
<td>0.52± 0.06</td>
</tr>
<tr>
<td>100</td>
<td>0.81± 0.05</td>
</tr>
<tr>
<td>C) PGF$_{2a}$ (µg/ml)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.52± 0.09</td>
</tr>
<tr>
<td>1</td>
<td>0.74± 0.02</td>
</tr>
<tr>
<td>10</td>
<td>1.52± 0.17</td>
</tr>
<tr>
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The trophoblastic tissues were prepared from term placenta. After incubation with a specified agent for
indicated hours, ir-OT in cultured media and
homogenized tissue was measured by radioimmunoassay.
The precise procedures are described in Materials and
Methods. The data presented are total content in media
and tissue (ng/g-tissue) and are expressed as the mean ±
SEM (n=4). Single (*) and double asterisks (**) indicate
P<0.05 and P<0.01 vs. control (§), respectively.
Fig. 1. Analysis by iron-exchange column chromatography (a) and bioactivity (b) of placental extract. (a) Placental extract was compared with synthetic OT on its elution profile on a carboxymethyl cellulose (CMC) column. Ammonium acetate buffer (pH 4.6) was used as eluent with its linear gradient concentration change (0.002-0.4 M). Fractionated samples were assayed by RIA for OT and the profiles for both substances were remarkably similar. (b) Biological activity of the extract was assayed by the isometric tension of uterine muscle. Synthetic OT (b-1;A; 1.6 ng/ml) and placental extract (b-1;B) showed potent activity, which was abolished by incubation with the antiserum for 2 days (b-1;C). Specific blockade by the antiserum was ascertained by the series of procedures shown in Fig.1-(b-2); the bioactivity of the placental extract (A) was abolished by the 2-day incubation with the antiserum (B) but not with NRS (C), whereas normal rabbit serum (NRS) and the antiserum (D and E respectively) did not have any contractile activity. The specificity of the antiserum is also shown in Fig.1-(b-3), where A and B represent synthetic OT and that incubated with antiserum for 2 days.

Fig. 2. Elution profile of the extract through Sephadex G-25 column chromatography (a) and bioactivity of its fraction (b). To compare the bioactivity and immunoreactivity of the extract, the fine procedure for extraction was employed as described in Materials and Methods. (a) The extracted powder was dissolved in 0.1 M acetic acid and applied to Sephadex G-25 column chromatography. The amount of amino acid in the eluted solution was measured by the Folin-Lowry method. The eluent was also measured for its ir-OT content in 7 fractions. (b) The biological activity of each fraction was assayed by isometric tension of uterine muscle. Two doses of synthetic OT were also applied to the apparatus for the control (C-1;0.3 IU/L and C-2;0.2 IU/L). As indicated in the record chart, the first, second, and fourth fractions had some contractile bioactivity.
Fig. 3. Gel-electrophoresis and Northern blotting of the molecular cloned substance. a) After repeated immunoscreening of a human placental cDNA library, the clone cDNA obtained was digested with EcoRI and electrophoresed on 1 % agarose gel. Its size was approximately 900 nucleotides (A). The right-side lane (M) is λ/Hind III:EcoRI double digest as size markers. Please refer to Materials and Methods for precise procedures. b) Northern blot hybridization analysis of RNA from human placenta and rat liver. Lanes 1 and 2: human placenta (third trimester), Lane 3: rat liver.

Fig. 4. Bioactivity of the molecular cloned substance. a) Effect of synthetic OT and recombinant substance on rat uterine contraction. A, B, C: synthetic OT (0.4, 0.2, 0.1 IU/L respectively); D: Recombinant substance from human placenta; E: Recombinant porcine FSH. b) Inhibitory effect of antiserum on bioactivity of recombinant substance. A: synthetic OT (0.2 IU/L); B: A+the antiserum (incubated for 1 day); C: the recombinant substance; D: C+the antiserum (incubated for 1 day).

uterine contractile activity: one is to determine the amino acid sequences of the bioactive fraction obtained from the placental extract through HPLC, the other to determine the bioactive portion of r-OT obtained in the immunoscreening described above. Since the placenta is closely attached to the uterine muscle and finishes its role with the completion of labor, it is an attractive hypothesis that a substance of placental origin plays a pivotal role in the mechanism of the initiation of labor. Recently Miller et al. [13] reported a potential paracrine role of OT synthesized by amnion, chorion, and decidua in the onset of human parturition. In addition to this scientific interest, the mechanism of labor onset is also important in clinical medicine, where tocolytic agents are critical for fetal well-being[14]. Still in progress is the search for the major contractile factor at the onset of and during delivery, by us and others.
References


