DETECTION OF THYROID HORMONES IN HUMAN EMBRYONIC CAVITIES DURING THE FIRST TRIMESTER OF PREGNANCY.

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ABSTRACT: Transfer of maternal thyroxine (T4) to the human fetus near term has recently been demonstrated. We investigated whether maternal thyroid hormone is available to the conceptus during the first trimester of pregnancy as well. Transvaginal ultrasound-guided puncture of the embryonic cavities was performed during the first trimester of pregnancy to obtain coelomic fluid between 6 and 11 weeks, and amniotic fluid between 8 and 11 weeks of pregnancy. T4 was found in coelomic fluid with mean values (± SEM) being 961 ± 193 pmol T4/L (747 ± 150 pg/mL). Concentrations increased both with gestational age and with rising maternal serum T4. Concentrations of 3,5,3'-triiodothyronine (T3) were at least 30 times lower, and those of 3,3',5'-triiodothyronine (rT3) four times higher, than coelomic fluid T4. Thyroxine and rT3 in amniotic fluid (8-11 weeks) were markedly lower than in the coelomic fluid, and T3 was undetectable. These results show that maternal thyroxine can cross the placental barrier as early as the second month of pregnancy. T4 from the coelomic fluid may reach the embryo via the yolk sac. This finding raises the possibility that the increase in maternal T4 occurring during the first trimester may be functionally important for the developing embryo, when its thyroid is not yet functioning.

In iodine deficient human populations, the frequency and the severity of neurological damage to the progeny is correlated to the degree of maternal hypothyroxinemia (1, 2). However, iodine supplementation only prevents the most severe developmental abnormalities if administered before conception or during the first trimester of pregnancy (3). These epidemiological findings raise important questions about the thyroid hormone status of the fetus early during pregnancy. The development of antenatal ultrasound-guided umbilical cord blood sampling has made it possible to study the changes in the concentration of fetal thyroid hormones during the second and third trimester of gestation (4), but blood-sampling using this method is not feasible before the 12th week of gestation. Therefore, little is known in man about thyroid hormones during the first-trimester of pregnancy, a period which precedes the beginning of fetal thyroid hormone synthesis and secretion (5).

The anatomy of the early gestational sac can be clearly visualized by means of transvaginal ultrasound (Figure 1). Up to 10 weeks of gestation, the amniotic cavity containing the developing embryo is completely surrounded by the exocoelomic cavity, containing the coelomic fluid, and by the placenta. Between the 5th and 12th week of gestation, the secondary yolk sac is floating freely in the exocoelomic cavity and is in continuity with the embryonic gut. After 10 weeks of gestation, the exocoelomic cavity disappears progressively, and two thirds of the placental villous membrane degenerates (6). The embryonic cavities can be successfully sampled under ultrasound guidance from the end of the 5th week of gestation onwards (7-9). The analysis of coelomic and amniotic fluids has demonstrated differences in the biochemical composition both between these fluids, and as compared with maternal serum (7, 9, 10). The results of these investigations clearly demonstrate that the coelomic fluid is an ultrafiltrate of the maternal serum, and that during the first trimester of pregnancy important nutrient transfer occurs between the mother and the fetus through the exocoelomic cavity and the yolk sac, which is reabsorbing the coelomic fluid. The coelomic fluid occupies a situation between the mother and the embryo, behind the placental barrier. These findings have led us to investigate the presence of thyroid hormones in the coelomic and amniotic fluids, at a stage of gestation when the fetal thyroid is not functioning, and to compare their levels with those in the maternal circulation.

SUBJECTS AND METHODS

Embryonic fluids and maternal serum were obtained from women undergoing termination of pregnancy for psycho-social reasons between 5.8 and 11 weeks of pregnancy. Informed written consent was obtained prior to surgery from each patient. Coelomic fluid (n=13) was obtained between 5.8 and 11 weeks of gestation by transvaginal puncture under ultrasonographic guidance as previously described (7-10), whereas samples of amniotic fluids (n=8) could only be collected from 8 weeks onwards. Both cavities were punctured independently. The exocoelomic cavity was punctured first and subsequently the amniotic cavity using a different needle. The procedure avoids contamination with maternal blood. Maternal blood (n=11) was taken from the forearm vein during the surgical procedure. All samples were stored at -70 °C. The sample volume available for the present study varied between 0.250 and 1.275 mL for the coelomic fluids and between 0.195 and 2.2 mL for the amniotic fluids. Coelomic and amniotic fluids were processed in a different laboratory from maternal blood samples without knowing the corresponding gestational age. The code was broken once all the data had been obtained.

Coelomic and amniotic fluids were processed for the determination of thyroxine (T4), 3,5,3'-triiodothyronine (T3) and 3,3',5'-triiodothyronine (rT3). All of each sample was purified individually following the method previously described (11) for plasma and tissue extracts, with modifications intended to improve recovery of the iodothyronines. The chloroform-methanol extraction, followed by back-extraction into aqueous phase was not used. Instead, after addition of the labelled recovery tracers, ethanol was added in a volume which was 2.5 times that of the sample. The samples were centrifuged and the ethanol extracts were purified through Biorad AG 1x2 columns, with further extensive purification as described (11). The iodothyronines were recovered in the 70 % acetic acid eluate, which was
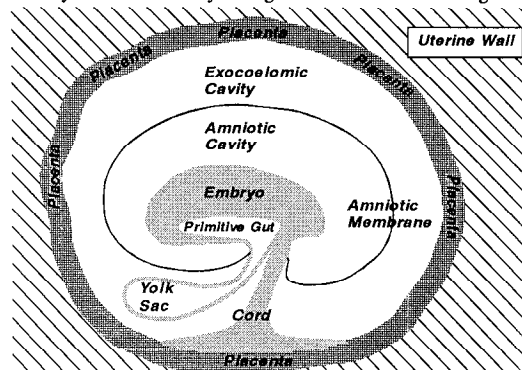

Figure 1. Schematic representation of the early gestational sac showing the anatomical relationships, during the first trimester of gestation, between maternal tissue, embryonic cavities and the embryo.

evaporated to dryness and reconstituted with 300 μL of RIA buffer, aliquots of which were used in the different highly sensitive RIAs, with cross-reactivities of analogues as described (11, 12). The minimum amounts detectable in the RIAs were 3.2 fmol of T4 (2.5 pg), 2.7 fmol of T3 (1.5 pg) and 1.8 fmol of rT3 (1 pg) per tube.

Extracts from four samples of coelomic fluid which were available in larger volumes were assayed in the T4 RIA at four different dilutions (5, 10, 20 and 40 μL) in duplicate, to confirm the absence of substances interfering in the RIA, and to verify that the behaviour of the extract in the assay was identical to that of the T4 standard. The remaining coelomic and the amniotic fluid extracts were assayed in duplicate at a single dilution using 40 μL for coelomic fluid and 50 μL for amniotic fluid. Single 100 μL aliquots were used for the T3 RIA and duplicate 45 μL aliquots were used for rT3. Concentrations were then calculated taking into consideration the recovery of the added tracer and the initial volume of sample.

Maternal sera were tested for total T4, "free" T4 (FT4), total T3, reverse T3 (rT3), and thyrotrophic hormone (TSH) by RIA, using Gammacoat from Clinical Assays for T4, T3, TSH, and FT4 (the two-step assay). Kits from Byk-Sangtec Diagnostica, (Germany) were used for rT3 and from Behring (Germany) for thyroxine binding globulin (TBG).

Statistical analysis were performed with SPSSPC program (Chicago, Illinois). Results are expressed as arithmetic means plus or minus one standard error of the mean (SEM).

Table 1 - Mean values (± SEM) of total and free (F) T4, total F3 and rT3, TBG and TSH in the maternal sera (A), and the normal range (B) for non-pregnant adults with the same assays.

	T4 nmol/L	F-T4 pmol/L	T3 nmol/L	rT3 pmol/L	TBG μmol/L	TSH mU/L
A	146±12	40.0±11.1	1.8±0.1	48.2±57	0.68±0.09	2.0±0.8
B	77-154	9.4-25.9	1.2-3.1	154-538	0.25-061	0.4-3.1

Multiply T4 and F-T4 by 0.078 to express concentrations in μg/dL and ng/dL, respectively; T3 by 65.2 and rT3 by 0.0652 to express results in ng/dL; TBG by 5.56 to convert to mg/dL.

RESULTS

Maternal thyroid status: Table 1 shows the mean circulating levels of T4, T3, FT4, rT3, TBG and TSH in the maternal sera. The mean T4 of sera obtained between 8-11 weeks of pregnancy was 180 ± 19 nmol / L (14.0 ± 1.5 μg / dL), and was significantly higher ($P < 0.05$) than in samples corresponding to 5.8 - 7 weeks, which was 126 ± 10 nmol / L (9.8 ± 0.8 μg / dL)). The mean FT4 value was higher, and the mean TSH was lower, in the 8-11 weeks group, but the differences did not reach statistical significance.

Coelomic fluid T4: Figure 2A shows the displacement of antibody-bound [125I] T4 using different amounts of purified T4, and the displacement obtained with different volumes of the purified extracts from four different samples of coelomic fluid. Serial dilutions of the extracts behaved as the T4 standard. The
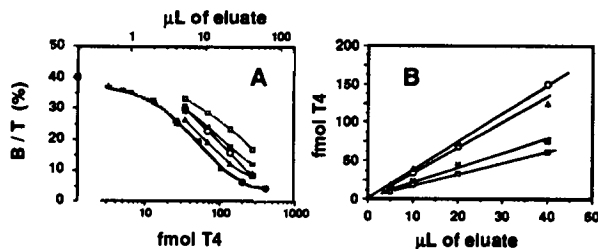
Figure 2. Panel A compares the standard curve (thick line) of the T4 RIA with serial dilutions of the purified extracts from four samples of coelomic fluid, purified as described in Subjects and Methods section. The ordinate shows the percentage of the total amount of radioactive antigen (T) that is specifically bound (B) in the RIA. The abscissa shows the amount of purified T4 added for the standard curve, or the volume of purified extract in the RIA assay tube, on logarithmic scale. Panel B shows the amounts of T4, as read off the T4 standard curve for each aliquot of an extract, plotted against the volume used in the assay.
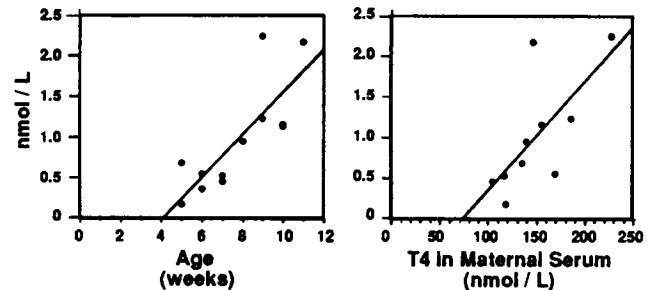
Figure 3. Concentrations of T4 in coelomic fluid are plotted as a function of gestational age in Panel A, and as a function of the maternal circulating T4 in Panel B. Positive correlations were found in both cases (r=0.803, $P < 0.01$ for A and r=0.699, $P < 0.05$ for B).

amount of T4, as read off the T4 standard curve, was proportional to the volume of extract (Fig 2B). Figure 3 shows the concentrations of T4 in the coelomic fluid as a function of gestational age (panel A) and of maternal serum T4 (panel B). In both cases, positive correlations were found. The overall mean concentration of T4 (± SEM) was 961 ± 193 pmol / L (747 ± 150 pg / mL). The mean values obtained when grouped according to gestational age were 519 ± 93 pmol / L (403 ± 72 pg / mL) for 5.8 - 8 weeks of gestation and 1581 ± 255 pmol / L (1229 ± 198 pg / mL) for 9-12 weeks ($P < 0.01$). Thyroxine in the coelomic fluid represented 0.14 % of total maternal serum T4 at 5.8 weeks of gestation and increased linearly up to 1.46 % at 11 weeks.

T3: Despite the fact that one third of the purified extract from each coelomic fluid was used in the T3 RIA, the amounts of this iodothyronine were below the limit of detection (2.7 fmol, 1.5 pg) in four samples, and barely detectable in the rest. The mean tentative value was 33 ± 13 pmol / L (18.5 ± 7.3 pg / mL), which is about thirty times lower than that of T4.

rT3: The concentration of rT3 was higher than expected, and the amount of rT3 in the aliquots initially used exceeded the highest standard (230 fmol, 130 pg). A second RIA was performed using only 5 μL of the final purified extract, in duplicate. Reliable rT3 values could be obtained for six samples of coelomic fluid, as no extract was left over from most samples. The mean value was 3.54 ± 1.19 nmol / L (1.97 ± 0.64 ng / mL). rT3 concentrations were 3.8 times the paired T4 concentrations. rT3 concentrations were positively correlated with duration of pregnancy, maternal T4, and T4 in coelomic fluid.

Amniotic fluid: T4 could only be detected in 3 samples for which the starting volume was at least 1.8 mL. The tentative mean value was 0.020 ± 0.005 nmol / L (16.0 ± 3.8 pg / mL). Although larger volumes of amniotic fluid are needed to give a reliable figure, it appears from these very preliminary measurements that the concentration of T4 in amniotic fluid is 50 times lower than in coelomic fluid. The concentration of T3 was below the limit of
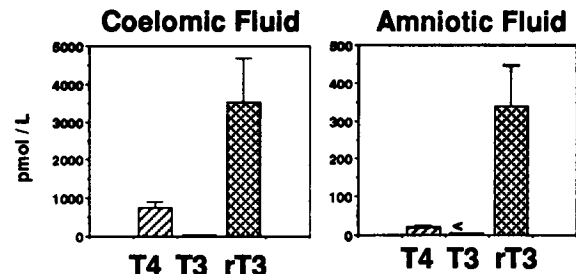
Figure 4. Comparison between mean (± SEM) concentrations of T4, T3, and rT3 in coelomic fluid (Panel A) and in amniotic fluid (Panel B). T3 in amniotic-fluid was below the limit of detection. Note the differences between the two panels in the scales used as ordinates .

detection in all samples. On the contrary, rT3 in amniotic fluid could be measured in all samples, the mean value being 0.399 ± 0.106 nmol / L (224 ± 59 pg / mL), which is at least 15 times the concentration of T4 in amniotic fluid, and 10 times lower than rT3 in coelomic fluid (Figure 4).

DISCUSSION

The mammalian placenta has long been considered virtually impermeable to thyroid hormones (13). As a consequence, it was thought that thyroid hormones i) would not be available to the fetus until the onset of its own thyroid function, and thence, ii) would not be necessary for normal early mammalian development. Present evidence strongly questions concept i), and suggests a possible revision of point ii).

i) Availability of maternal thyroid hormone before onset of thyroid function: Experimental studies in rats have shown that, unless the mother is hypothyroid (11), both T4 and T3 are available to the embryo before onset of its own thyroid function (14, 15), and that the maternal transfer of T4 continues until term and protects the brain of a hypothyroid fetus from T3 deficiency (16). Early availability of maternal thyroid hormones has also been shown for a non-mammalian species (17).

Information for man is less complete. Fetal blood can be obtained by cardiac puncture or cordocentesis from the 12th week of gestation onwards, allowing the study of fetal serum thyroid hormone evolution (4, 18). From such studies, however, the date of onset of fetal thyroid function cannot be assessed, as neither the extent of the possible maternal contribution to the fetal circulating pool, or its evolution with gestational age, are known. As recently discussed (5), secretion of the iodothyronines is believed to start at midgestation (18-22 weeks), after development of the median eminence portal system, when there is a generalized maturation of anterior pituitary secretory cells and a concomitant sudden increase in fetal plasma T4 (19). Some reports suggest that human thyroids in organ culture have the capacity to synthesize thyroxine by the 10-12th weeks of gestation (20), but findings *in vivo* indicate 17 - 19 weeks as the earliest date when labeled iodothyronines are found in the fetal thyroid after administration of radioiodine to the mother (21).

Transfer of maternal T4 and T3 was found using radiolabeled iodothyronines in normal pregnancies at term (22, 23), and has recently been quantified in babies born with total organification defect, whose cord-blood T4 was 20-60 % of normal newborn values (24). Transfer of labeled hormones from the mother to the fetal circulation was also shown earlier in pregnancy (11-25 weeks of gestation), but could not be quantified (25). T4 and T3 are found in early human embryonic and fetal tissues, particularly in the brain (26, 27). The earliest age studied was 9 weeks of gestation, clearly indicating that maternal thyroid hormones already reach the conceptus during the third month of pregnancy.

Present results are the first demonstration that maternal T4 reaches an embryonic cavity as early as 3.8 weeks after conception (5.8 weeks after the last menstrual period), when there is little doubt that the fetal thyroid is not yet functioning. The concentration of T4 in the coelomic fluid is moreover related to the maternal thyroxinemia, supporting the maternal origin of the iodothyronine. Despite only representing from 0.14% up to 1.4% of the maternal T4, coelomic T4 is 10 to 100 times more elevated than the corresponding maternal free T4 at 5.8 and 11 weeks respectively. The preliminary results in the 8-11 weeks amniotic fluid samples agree with previous reports (28, 29) on the presence of T4 and rT3 in amniotic fluid samples obtained from 11 to 30 weeks of gestation. However, concentrations of both T4 and rT3 were markedly higher in the present coelomic fluid samples than in age-paired amniotic fluids.

T4 in coelomic fluid could be bound both to transthyretin (TTR, a T4 protein carrier) and to albumin, both being present in in the exocoelomic cavity. But while TTR increases with gestational age from from 0.18 μmol / L (1 mg /dL) at 6 weeks of gestation to about 1.08 μmol / L (6.0 mg / dL) at 9 weeks, albumin decreases (9). Transfer of T4 to the embryo could be facilitated by TTR synthesized by the yolk sac epithelium and secreted preferentially towards the embryo (30). The mechanism of transfer would be similar to the one described for the transport of T4 into the brain, via TTR synthesized in the choroid plexus (31). In man, in vitro synthesis of TTR by the yolk sac was shown at 5.5 weeks of gestation (32). The TTR gene is clearly expressed in the choroid plexus by 8 weeks of gestation, which is the earliest age studied, and only later in other embryonic tissues, such as the gut and pancreas (33). Thus, TTR is expressed very early in pregnancy at two sites which are important for the transport of T4 from the coelomic fluid to the embryo, and from the embryo to the brain. TTR was well preserved during evolution, especially in the choroid plexus, suggesting an important role for this protein either in the transport of T4 or vitamin A, or both (32). A recent paper (35), however, reports phenotypically normal mutant mice with disruption of the TTR gene, questioning the actual importance of TTR, at least in mice.

The presence of T4 in coelomic fluid suggests that thyroid hormones may reach the developing brain as early as the second month of gestation, and confirms the maternal source of the thyroid hormone found in the brain of early fetuses (26, 27). The concentrations of T3 in coelomic fluid is much lower than that of T4, and undetectable in age-paired amniotic fluids. Undetectable concentrations have also been reported by others in second trimester amniotic fluid (28, 29), and fetal blood (4, 18). Despite this, T3 is found by 9 weeks of gestation in the fetal brain, and brain nuclei. In liver, lung and heart, only T4 was found throughout the second trimester (26); although T3 could be demonstrated at earlier ages in lung nuclear extracts (27). 5 and 5'-iodothyronine deiodinase activities have been reported in human fetal brain at 11-25 weeks of gestation (36), but we are unaware of information from early first-trimester embryos.

ii) Thyroid hormones and early development: The presence of thyroxine in early embryonic cavities and fetal tissues does not *per se* constitute proof that thyroid hormones are playing a functionally significant role early in development. Available evidence is, however, compatible with this possibility. Nuclear thyroid hormone receptors are found in rat embryonic and fetal tissues before onset of fetal thyroid function (37), with the c-erbA α1 isoform already expressed early in development, when the neural tube is formed (38). Very early expression of the c-erbA α1 isoform has also been reported for a non-mammalian species (39). Early rat embryos cultured in serum from both hypothyroid and hyperthyroid rats show a marked increase in malformations, including neural abnormalities (40). In man, nuclear receptors for thyroid hormones have been measured between 9 and 18 weeks of gestation (26, 27). Receptor occupancy by T3 was 25 % in brain, with equimolar amounts of T4 being also found in the nuclear fraction (27). Low levels of maternal T4 during pregnancy appear to impair the mental development of the progeny (41, 42). As indicated in the introduction, correction of iodine deficiency before conception or during the first trimester is necessary to prevent the central nervous system damage seen in neurological cretinism (1-3). The suggested timing of some of the insults to the developing brain corresponds to the end of the first trimester, or the beginning of the second (43).

Possible clinical implications: During pregnancy, maternal thyroid function shows well defined changes (44, 45). During the first trimester, there is a considerable increase in the circulating levels of TBG due to increased estrogen production. Consequently, total T4 and T3 increase. Moreover, although the thyrotrophic potency of human chorionic gonadotrophin (hCG) is low as compared to TSH, the very high levels of hCG are enough to significantly stimulate the thyroid gland. Maternal TSH decreases and the normal control of thyroid function by TSH

is superseded by hCG; FT4 levels peak at 10 weeks of gestation, when maternal hCG levels are highest. This might represent a further example, if thyroid hormone is necessary during embryogenesis, of a maternal endocrine gland which is being controlled by the conceptus for its own benefit. The present data suggest, although do not prove, that this (hCG-dependent) increase in T4 and FT4 during the first trimester would ensure an adequate supply of T4 during the initial phase of gestation, when the fetal thyroid gland is not yet active. The present results also point to the possible importance of investigating and adjusting maternal thyroid hormone levels during the first trimester of pregnancy. Clinical studies have demonstrated that hypothyroid women need to increase their thyroxine supplementation dose by 50 % during pregnancy (46). Even in situations other than iodine deficiency, failure of the maternal thyroid gland to adapt to hormonal changes observed in normal pregnant women may prevent the increase in T4 levels which is normally observed during the first trimester of pregnancy, and thus affect the availability of thyroid hormone to the developing embryo.

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