Plasma Progesterone, Blastocyst Steriodogenesis and Blastocyst Survival in Rats with Altered Thyroid Status

James P. Holland, Richard Brooks and Erich Weidenbener
Department of Biology
Indiana University
Bloomington, Indiana 47405

Introduction

Studies in our laboratory continue to investigate the mechanism by means of which thyroid hormone influences reproductive physiology in the female rat. Thyroid hormone has been reported to exert effects upon the reproductive system and pregnancy in many types of animals, including human beings. These findings have been reviewed by Leathem (11). However, the mechanisms by means of which thyroid hormone exerts these effects have not been elucidated. In our laboratory, earlier investigations of thyroidal influences upon reproduction have utilized the technique of experimentally delayed implantation of blastocyst in rats (4), which allows the investigator to control some of the variables which one encounters in studies of reproduction. For example, this technique allows the investigator to control the levels of sex steroids available during early pregnancy, allows control of the time of implantation, and allows the separation of progesterone-dependent effects from estrogen-dependent effects. Our earlier investigations (8, 9) using rats demonstrated that thyroxine, in dosages as low as 8 ug per day, can compensate for progesterone deficiency during the progesterone-dependent maintenance period of experimentally induced delayed blastocytes. The opposite effect was caused by surgical thyroidectomy which further intensifies the detrimental effects of progesterone deficiency upon the survival of blastocysts. Our studies have further demonstrated that the thyroidal effect upon progesterone-dependent blastocyst survival is exerted by means of direct effects upon the blastocyst as well as by means of effects upon the uterus which indirectly effect the blastocyst. For example, thyroid hormone was demonstrated to stimulate RNA and protein synthesis in the blastocyst (1) and was demonstrated to stimulate the activity of a uterine enzyme associated with blastocyst survival (9). In the present investigations there is a continued examination of a direct effect and an indirect effect of thyroid hormone upon the rat blastocyst. For a direct effect the influence of thyroid hormone upon blastocyst steroidogenesis was examined, and for an indirect effect the influence of thyroid hormone upon plasma levels of progesterone was examined.

Materials and Methods

Sprague-Dawley-derived female albino rats (Harlan Industries, Cumberland, Ind.) between 60 and 120 days old were maintained on Wayne Laboratory Chow and tap water ad libitum. All rats were housed in an animal room at 24°C with a daily illumination schedule of 14 hours of light and 10 hours of darkness. Hyperthyrodism was induced by daily injection of 48 μg L-thyroxine (Sigma Chemical Co.) beginning at least ten prior to the experiment. Surgical thyroidectomies were performed through a mid-ventral incision in the neck at least four weeks prior to the experiment.

Blastocyst Cytochemistry

Female rats showing a proestrus or estrus vaginal smear were placed overnight in cages with adult male rats. Insemination was confirmed on the following morning by the presence of spermatozoa in the vaginal smear and this was designated as Day 1 of pregnancy. Experimental delay of implantation was accomplished by ovariectomy

on Day 3 of pregnancy and daily injections of 0.4 mg of progestrone (the deficiency dosage as determined in earlier studies; 8, 9). Blastocysts were flushed from the uteri excised from control, hyperthyroid, and hypothroid rats on either Day 5 of pregnancy (normal, non-delayed blastocysts) or on Day 8 of pregnancy (the final day of the progesterone-dependent delay period during delay of implantation). Using a one-milliliter syringe filled with 0.1 M phosphate buffer (pH 7.4) and fitted with a 25 gauge needle, the blastocysts were washed and flushed into depression slides. Blastocyst cytochemistry for the determination of 3β hydroxysteriod dehydrogenase was conducted according to the procedure of Dey and Dickman (5). For each blastocyst cytochemistry experiment, the incubation medium was freshly prepared. This medium was prepared by adding the following to 9.6 ml of 0.1 M phosphate buffer (pH 7.4): 1.8 mg dehydroepiandrosterone, 4.5 mg nicotinomide adenine dinucleotide (NAD), and 2 mg Nitro Blue tetrazolium dissolved in a minimal amount of dimethyl formamide (all obtained from Sigma Chem. Co., St. Louis, MO.). An aliquot of 0.5 ml of the incubation medium was placed in each depression of depression slides. Three to four blastocysts were placed into the medium of each depression; each depression slide was then placed in a Petri dish containing moistened filter paper and these dishes were incubated at 37 °C for three hours. As controls, some depressions did not contain the substrate dehydroepiandrosterone. After three hours the blastocysts were removed from the depression slides, placed on microscope slides, and at 100X and 430X magnification they were analyzed for intensity of the formazan reaction and were photographed.

Progesterone Radioimmunoassay

Control, hyperthyroid, and hypothyroid rats were ovariectomized and injected daily with 0.4 mg progesterone for five days in order to simulate the progesterone maintenance period in the delayed implantation experiments. On the sixth day (comparable to Day 8, the final day of blastocyst delay in the delayed implantation experiments) the rats were anesthesized with ether and blood samples were removed by means of cardiac puncture. Heparin dissolved in physiological saline was used as anticoagulant.

Radioimmunoassay of plasma was accomplished using Coat-A-Count, solid phase ^{125}I radioimmunoassay kits prepared by Diagnostic Products Corp., Los Angeles, Calif. (6). Duplicate aliquots of 100 ul of each plasma sample were used for the determinations, incubation time was three hours at room temperature, and the tubes were counted for one minute in a Beckman Gamma 4000 gamma counter. Corrections were made for non-specific binding, a seven-point standard curve was established, and the progesterone levels in the plasma samples were expressed as ng per milliliter. The assay is sensitive to a minimum of 0.05 ng per milliliter.

Results

Table I summarizes the evaluations of the cytochemical reaction for 3 β-hydroxysteriod dehydrogenase in non-delayed blastocysts which were flushed from the uteri of control, hyperthyroid, and hypothyroid rats on Day 5 of pregnancy. High amounts of the enzymatic reaction were present in all of these blastocysts, and there were no differences between blastocysts which were obtained from rats of different thyroid states. As a control for the reaction, blastocysts which were incubated in medium without the dehydroepiandrosterone substrate did not show the darkening indicative of the enzymatic reaction.

Table 2 summarizes the evaluations of the cytochemical reaction for 3 β-hydroxysteroid dehydrogenase in delayed blastocysts (maintained on a deficiency dosage of progesterone) which were flushed from the uteri of control, hyperthyroid, and

TABLE 1. Summary of Histochemical Determinations of 3 β-OH Steroid Dehydrogenase in Five-day Blastocysts Obtained from Rats of Different Thyroid States (Intact Ovaries—No Exogenous Progesterone)

Treatment	No. Blastocysts Examined	Enzyme Reaction in Trophoblast	Enzyme Reaction in Inner Cell Mass
Euthyroid	26	+ + +	+ + +
Hyperthyroid	24	+ + +	+ + +
Hypothyroid	17	+ + +	+ + +

Blastocysts were incubated for three hours at 37°C in 0.1 M phosphate buffer containing dehydroepiandrosterone, NAD, and Nitro Blue tetrazolium.

hypothyroid rats on Day 8 of pregnancy. The blastocysts from control and hyperthyroid rats showed approximately the same levels of enzyme activity, except for possibly higher activity in the inner cell mass area of those from hyperthyroid rats. The blastocysts

TABLE 2. Summary of Histochemical Determinations of 3 β-OH Steroid Dehyrogenase in Eight-day Blastocysts Obtained from Rats of Different Thyroid States (Experimentally Delayed Blastocysts)

Treatment	No. Blastocysts Examined	Enzyme Reaction in Trophoblast	Enzyme Reaction in Inner Cell Mass
Euthyroid + 0.4 mg Prog.	28	+ + +	+
Hyperthyroid + 0.4 mg Prog.	55	+ + +	+ + +
Hypothyroid + 0.4 mg Prog.	16	+	-

All animals were ovariectomized on Day 3 of pregnancy and maintained with progesterone until autopsy on Day 8.

Blastocysts were incubated for three hours at 37°C in 0.1 M phosphate buffer containing dehydroepiandrosterone, NAD, and Nitro Blue tetrazolium.

from the hypothyroid rats, however, showed much lower levels of the enzyme activity and some of these blastocysts were entirely devoid of the enzyme activity. Again, the blastocysts which were incubated in medium without the dehydroepiandrosterone did not show darkening.

Table 3 contains the data from the ^{125}I radioimmunoassay determinations of plasma

TABLE 3. Effect of Altered Thyroid States Upon Plasma Progesterone Levels in Ovariectomized Rats Injected Daily with 0.4 mg Progesterone for Five Days

Treatment	No. Rats	Plasma Progesterone + (ng/ml)
Euthyroid	8	5.27
Hyperthyroid	8	4.55
Hypothyroid	8	9.32*

+Corrected to uniform body weights.

Progesterone determinations by means of radioimmunoassay (Diagnostic Products Corporation).

*Significantly different from euthyroid and hyperthyroid ($P < 0.05$) as determined by Tukey and Scheffe analysis.

progesterone in rats of different thyroid states. It can be seen that there was no difference in plasma progesterone level in control and hyperthyroid rats. On the other hand, the hypothyroid rats had a significantly higher plasma progesterone level than controls (9.32 ng/ml and 5.27 ng/ml, respectively).

Discussion

The survival of blastocysts in rats which are ovariectomized on the third day of pregnancy is progesterone-dependent. Implantation and further development of these blastocysts are "delayed" since ovariectomy removes the source of estrogen which is essential for the implantation process (13). The normal daily maintenance dose of progesterone during delay is 2.0 mg; a deficiency dose of 0.4 mg/day causes a significant decrease in the number of surviving blastocysts (10). Thyroid hormone has been demonstrated to exert important effects upon delayed blastocysts during a deficiency of progesterone. Our earlier studies have shown that these thyroidal effects may be exerted directly upon the blastocyst or indirectly by means of altered uterine physiology. The full extent and mechanisms of the direct and indirect effects of thyroid hormone have not been elucidated. The present studies were conducted to determine whether maternal thyroid activity can alter the activity of an enzyme in the blastocyst which is important for progesterone synthesis and whether thyroid hormone influences the plasma levels of progesterone which may influence uterine physiology.

Using cytochemical determinations of 3 β-hydroxysteroid dehydrogenase, a key enzyme in steroidogenesis, investigators (5) have demonstrated that the pre-implantation blastocysts of the rat and the rabbit synthesize steroid hormones. It has also been suggested that blastocysts of the rabbit accumulate steroids from the uterine fluid (2). In the present studies the five-day blastocysts from normal, intact rats all showed high levels of the 3 β-hydroxysteriod dehydrogenase regardless of the thyroid status of the mother. This is not surprising since our data from earlier experiments have all indicated that thyroidal effects only become important during progesterone deficiency. No such deficiency existed in these intact rats. On the other hand, the delayed blastocysts in hypothyroid rats which had been maintained on a deficiency dosage of progesterone showed lower intensity of the enzyme reaction. This finding correlates well with our earlier studies. The hypothyroid, progesterone-deficient rats comprise the group which had the lowest survival of blastocysts (8, 10), and these blastocysts showed the lowest amount of protein synthesis (1). However, the ability of hyperthyroidism to overcome the detrimental effects of progesterone deficiency cannot be explained by means of the 3 β-hydroxysteroid dehydrogenase studies since blastocysts from control and hyperthyroid rats showed approximately the same levels of the enzymatic reaction. In order to more clearly determine whether thyroid hormone influences steroidogenesis in the rat blastocyst, we are conducting *in vitro* steroidogenesis experiments with long-term (four days) incubated blastocysts using NCTC-135 (GIBCO) nutrient medium which is changed daily and analyzed for progesterone by means of radioimmunoassay. Recently, McCormack (12) reported *in vitro* studies of rat blastocyst steroidogenesis using this procedure. They found that the blastocyst production of progesterone was low and variable.

An indirect route by means of which thyroid activity may influence blastocyst survival is by altering the metabolism of progesterone and its action upon the uterus. Bradlow et al. (3) reported that thyroid hormone alters the activity of enzymes that transform progesterone *in vivo* in hyperthyroid human beings, resulting in a shift toward the production of 5 α-reduced metabolites of progesterone. Our preliminary studies reported here show that in rats which were treated with progesterone in a manner to simulate the delayed implantation studies, hypothyroid rats have a significantly higher

plasma progesterone level than do control or hyperthyroid rats. Since the hypothyroid rats are the ones with the lowest survival of blastocysts, the higher plasma progesterone level in these rats may reflect an altered metabolism (utilization, degradation, excretion, etc.) of progesterone which has an overall negative effect upon uterine physiology and blastocyst survival. Again, the beneficial effects of thyroid hormone cannot be explained by the present data. Gas chromatographic studies of progesterone metabolism in rats of different thyroid states are underway to further examine the conversion of progesterone to related compounds and the excretion patterns of progesterone metabolites. Also, uterine progesterone receptor binding studies should be helpful in approaching firm conclusions concerning the effects of thyroid hormone which are exerted by way of the uterus. Receptors for thyroid hormone were recently identified in the rat uterus (7); therefore, thyroidal effects upon the uterus are expected to be significant.

Collectively the present studies concerning blastocyst cytochemistry and plasma progesterone levels are generally supportive of our earlier findings that thyroid hormone can influence blastocyst survival by means of direct effects upon the blastocyst and by methods which influence uterine physiology. Further studies are in progress in order to more clearly determine the mechanisms which are involved.

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