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Restoration of corpus luteum angiogenesis in immature hypothyroid *rdw* rats after thyroxine treatment: Morphologic and molecular evidence

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ABSTRACT

Thyroxine (T4) plus gonadotropins might stimulate ovarian follicular angiogenesis in immature infertile hypothyroid *rdw* rats by upregulating mRNA expression of major angiogenic factors. Development of growing corpus luteum (CL) is strongly related to angiogenesis and to morphofunctional development of microcirculation. Our aim was to investigate if T4 is involved in CL angiogenesis and in the activation of capillary cells and angiogenic factors after ovulation in a spontaneous model of hypothyroidism, the *rdw* rat. *Rdw* rats were treated with T4 plus gonadotropins (equine chorionic gonadotropin plus human chorionic gonadotropin; eCG+hCG) or gonadotropins alone in order to evaluate the effects of T4 on early luteal angiogenesis, on microvascular cells and on expression of major growth factors which are involved in the regulation of angiogenesis. Wistar-Imamichi rats treated with gonadotropins were used as controls. The ovaries were collected 4 days after hCG administration and analyzed using morphologic and molecular approaches. Thyroxine plus gonadotropins stimulated the growth of CLs and follicles as in controls, differently from *rdw* rats treated only with gonadotropins, in which CLs were not found and only small follicles, often atretic, could be recognized. In T4 plus gonadotropin-treated *rdw* rats CLs showed increased microvasculature, numerous activated capillaries characterized by sprouting and other angiogenic figures, and associated pericytes. Quantitative analysis revealed that the number of pericytes in T4 plus gonadotropin-treated *rdw* rats was comparable with that found in control rats and was significantly higher than that found in gonadotropin-treated *rdw* rats. The mRNA expression of vascular endothelial growth factor and basic fibroblast growth factor was significantly higher in control rats and in T4 plus gonadotropin-treated *rdw* rats than in gonadotropin-treated *rdw* rats. mRNA expression of tumor necrosis factor α, transforming growth factor β, and epidermal growth factor did not show significant changes. Our data originally demonstrated that T4 promoted the growth of an active microcirculation in developing CLs of gonadotropin-primed hypothyroid *rdw* rats, mainly by inducing sprouting angiogenesis, pericyte recruitment, and upregulation of mRNA expression of vascular endothelial growth factor and basic fibroblast growth factor. In conclusion, we suggest that T4 plays a key role in restoring luteal angiogenesis in ovaries of immature hypothyroid *rdw* rats.

1. Introduction

Folliculogenesis, ovulation, and corpus luteum (CL) formation are cyclically related to phenomena of microvascular growth, remodeling and regression of blood vessels, and therefore to specific functional changes of the ovarian microcirculation. All these adaptations are fundamental to ensure fertility and normal ovarian function [1–5].

Angiogenic remodeling is particularly intense after ovulation, when new vessels develop from the pre-existing

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thecal vasculature to sustain the development of the initial CL [6–12]. CL is a highly vascularized structure that receives the greatest blood flow per unit of tissue of any organ in the body [12]. As a consequence, the rate of tissue growth and cell proliferation in growing CLs is equal to or even greater than that of the most rapidly growing and dangerous tumors [13]. Pericytes represent a large proportion of proliferating cells in growing CLs [14] and they act as guiding structures aiding the outgrowth of endothelial cells during luteal development [15]. The CL development and functionality is also ensured by the release into luteal cells of intercellular vesicles—secreted by vascular pericytes—containing a high quantity of Thy-1^{+} differentiation protein, a morphoregulatory molecule associated with cell differentiation [16].

The angiogenic process is finely regulated by several hormones and growth factors that act as either stimulatory or inhibitory factors [2,3]. In the mammalian ovary, the main regulators of angiogenesis are represented by gonadotropins (follicle [FL] stimulating hormone [FSH] and luteinizing hormone [LH]) and several angiogenic factors including vascular endothelial growth factor (VEGF) [17,18]. The ovary locally produces several other growth factors with angiogenic properties such as: transforming growth factor β (TGFβ) epidermal growth factor (EGF) and tumor necrosis factor (TNF)-α [17]. Recent findings demonstrated that the thyroid hormone, thyroxine (T4), also plays a key role in ovarian FL angiogenesis [19]. It is well known that hypothyroidism is associated with infertility problems, ranging from anovulatory cycles, and sterility to abortion in many mammals and humans [20–22]. Such anomalies could be partially or totally restored by T4 administration [23] or by a combined therapy with T4 including gonadotropins, as reported in hypothyroid women [21] and in a well established model of congenital hypothyroidism, the *rdw* rat [19,24,25].

Numerous studies highlighted that T4 is actively involved in coronary and brain angiogenesis [26–28]. The mechanism of thyroid hormone-induced angiogenesis is initiated with integrin αVβ3 receptor [29] after T4 binding and involves the secretion of basic fibroblast growth factor (bFGF) and VEGF by endothelial cells, as recently reviewed [30]. In the ovary of immature female *rdw* rats, T4 markedly improved the development of follicular microvasculature, especially in the presence of eCG, by regulating the gene expression of some growth factors involved in the regulation of angiogenesis, such as VEGF, TNFα, bFGF, and of their receptors [19]. However, although it was demonstrated that T4 therapy supports the formation of functional CL and the establishment of normal pregnancy in hypothyroid rodents [31], the effect of this hormone on ovarian luteal angiogenesis has not yet been addressed.

In this study we tested the hypothesis that T4 might mediate the development of CL microcirculation in immature infertile hypothyroid *rdw* rat ovaries and mRNA expression levels of main ovarian angiogenic growth factors such as VEGF, TGFβ-1, bFGF, EGF, and TNFα.

To this aim, we performed: (1) a structural and ultrastructural study by light microscopy (LM), transmission electron microscopy (TEM), and scanning electron microscopy (SEM) of vascular corrosion casts (vcc) to analyze the ovarian microvasculature, evaluated especially in terms of capillary activation and pericyte recruitment [1,4,19]; (2) a quantitative analysis to determine the number of pericytes in follicular and luteal capillaries [5]; (3) a semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) to analyze the mRNA expression of VEGF, TGFβ-1, bFGF, EGF, and TNFα, all involved in gonadotropin-stimulated luteal angiogenesis [2,3,13,19,32].

2. Materials and methods

2.1. Animals

Infertile immature hypothyroid *rdw* rats (N = 12; six per each experimental group), and their normal littermates (Wistar-Imamichi rats; [control] N = 4) were used. Animals were produced and maintained as previously described [19,24,25,31]. The *rdw* mutants were distinguished according to low body weight, retarded development of ears, and small body size at approximately 2 weeks of age. This study conformed to the Ethics Committee for Care and Use of Laboratory Animals for Biomedical Research of the Faculty of Agricultural Sciences, Tohoku University, Japan and to the E.C. regulation on this matter.

2.2. Experimental groups and hormonal treatments

Group 1 included Wistar-Imamichi rats, treated with eCG and hCG to induce superovulation and CL formation, used as control rats. Female immature *rdw* rats were divided randomly and treated as follows [24]: group 2, eCG and hCG; group 3, T4 and eCG and hCG.

eCG (10 IU) (Sankyo Kabu Company, Tokyo) was injected subcutaneously on Day 28 after birth; hCG (10 IU) (Sankyo Kabu Company, Tokyo) was given intraperitoneally (ip) 54 hours after eCG administration. Thyroxine (L-thyroxine, Sigma Chemical Company, St. Louis, MO, USA) was administered intraperitoneally (ip) once a day at a dose of 10 mg per 100 g of body weight, from Day 21 to Day 30 [19]. Thyroxine was dissolved in 2 mol NaOH and prepared in physiological saline solution (pH 8.3). The animals were sacrificed 4 days after hCG administration to study CL angiogenesis in the period of maximal expression (i.e., in the developing CL).

2.3. Experimental design

2.3.1. Morphologic study

2.3.1.1. Light microscopy and TEM. To assess the cellular morphologic changes of CL formation and ovarian microvasculature, LM (in semithin sections) and TEM (in ultrathin sections) studies were conducted as described previously [4,5,19]. Budding and sprouting (activated) capillaries were considered proliferative (angiogenic), and regular capillaries were considered quiescent. Proliferating and quiescent capillaries were classified on the basis of the whole capillary vessel ultrastructure, giving a special attention to the endothelial cell morphology (shape, plasma membrane specialization, nucleus morphology, and cytoplasmic