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Human ovarian theca cells are a source of renin

(ovary/renin–angiotensin)

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ABSTRACT **Human ovarian follicular fluid contains renin-like activity. In normal women, circulating levels of prorenin, the biosynthetic precursor of renin (EC 3.4.23.15), change in parallel with changes in progesterone during the menstrual cycle. Therefore, the ovary has been implicated as a source of plasma prorenin. In the present studies, we report the finding of high concentrations of prorenin in human ovarian follicular fluid (3000 ng·ml^{-1}·hr^{-1} vs. 10–40 ng·ml^{-1}·hr^{-1} in normal human plasma) obtained from follicles of women prepared for *in vitro* fertilization. The inactive renin-like enzyme was identified as prorenin by its activation characteristics, its molecular weight of 47,000, which is the same as that for recombinant prorenin, and its cross-reactivity with human renal renin antibodies. Culture of isolated human theca cells and isolated granulosa cells indicated that prorenin is secreted by theca cells but not by granulosa cells. Prorenin production by theca cells peaked during the first 10 days of culture and gradually decreased by 17 days. Active renin levels were 10% or less of the prorenin levels. Prorenin was barely detectable in medium from granulosa cells cultured for 24 days. Immunohistochemical staining of human ovaries (n = 5) with anti-human renin antibody demonstrated the presence of renin primarily in theca cells. These studies suggest that the theca cell is the source of the large quantities of prorenin in human ovarian follicular fluid.**

The production of the enzyme renin (EC 3.4.23.15) by the kidney regulates a cascade of events in the circulation that ultimately generate angiotensin II (AII). This octapeptide vasoconstricts vascular smooth muscle and stimulates aldosterone biosynthesis, thereby establishing the renin–angiotensin system as a major determinant of blood pressure (1). However, extrarenal renin–angiotensin systems may have other diverse physiologic effects (2). In normal humans, more than one-half of the circulating renin exists as the inactive biosynthetic precursor prorenin (3). Although the kidney appears to be a primary source of the prorenin as well as active renin in plasma, immunohistochemical and *in situ* hybridization studies have identified potential extrarenal sites of renin production. These include pituitary, adrenal, testis, uterus, and chorion-decidua (2, 4–8). Human chorion-decidua produces and secretes primarily prorenin (7, 8). Whether active renin or prorenin is the major form of renin produced by other extrarenal sources is unknown. However, several years after nephrectomy, plasma prorenin levels, but not active renin levels, increase in anephric subjects (9). Thus, in the absence of the kidney, extrarenal sources are capable of secreting prorenin. While renal renin production may regulate overall vascular homeostasis, regulation at a paracrine or autocrine level has been assigned to these local renin–angiotensin systems (2). For example, a pituitary and hypothalamic renin system appears to regulate prolactin and antidiuretic hormone production and to control dipsogenic behavior (10, 11). An adrenal renin system exists that regulates aldosterone production by the zona glomerulosa (12).

Recently, renin-like activity and even larger quantities of a trypsin-activatable renin have been found in human ovarian follicular fluid (13, 14). Prior to activation this renin cross-reacted with antibody generated against a peptide consisting of the 12 carboxyl-terminal amino acids of the human renin prosequence, confirming its identity with prorenin (14).

The objective of the present investigation was to localize the site of renin production in the human ovary.

METHODS

Four women were treated with human menopausal gonadotropin (Pergonal, 2 amps daily for 5 days; clomiphene, 100 mg daily for 5 days) on days 3–9 of their menstrual cycle in preparation for *in vitro* fertilization (IVF). On days 12–16 of the cycle after human chorionic gonadotropin administration, ovarian follicular fluid was aspirated from mature follicles. Plasma was also obtained from these subjects for measurement of renin. Active renin and prorenin concentrations were measured as described (15). Reversible acid activation of follicular fluid prorenin was tested (15) and trypsin activation of renin (14) was compared to results obtained with acid activation.

The immunoblot methodology has been described in detail (16). Rabbit antiserum developed against pure human renin (16) was used to detect immunoreactive forms of renin. This antiserum had a 50% inhibitory titer of 1:30,000 against 1×10^{-4} Goldblatt unit of MRC human renal renin and pure renal renin. Immunohistochemical staining of normal human kidney using this antibody demonstrated staining localized to the juxtaglomerular area only. Furthermore, the antibody did not cross-react with trypsin, pepsin, human renal kallikrein, or cathepsin D.

Granulosa and theca cells were isolated and cultured from premenopausal ovaries obtained in the course of benign pelvic surgery by the method of Lobb and Dorrington (17). Granulosa cells were obtained by gentle scraping of the inner surface of a follicle, centrifuged, and plated (10^5 cells per ml) in Dulbecco's modified Eagle's medium and Ham's F-12 medium (1:1) containing 10% fetal calf serum. The theca layer was peeled from the stroma, digested, centrifuged, and plated. Stimulated granulosa cells were also obtained after centrifugation of ovarian follicular fluid obtained from IVF patients. In addition to active renin and prorenin, 4-androstene-3,17-dione and progesterone (18) were measured in the culture medium.

Immunohistochemical studies using the anti-human renal renin antibody were performed on normal ovarian tissue

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Abbreviations: AII, angiotensin II; IVF, *in vitro* fertilization.

obtained from five premenopausal women undergoing ovariectomy for ovarian cysts. Ovarian tissue was fixed in 10% formalin and embedded in paraffin. After incubation with rabbit anti-human renal renin antiserum (1:100, 48 hr at 4°C), sections were developed with avidin–biotin complex system (Vector Laboratories).

RESULTS

Human ovarian follicular fluid contained nearly 20 times the normal plasma levels of active renin and 100 times the plasma levels of prorenin (Table 1). Acid treatment or trypsin treatment of follicular fluid yielded similar values of prorenin. Acid activation of follicular fluid prorenin was reversible. Reexposure to acid renewed enzyme activity. Anti-human renal renin antibody (10^4 dilution) inhibited 50% of the enzyme activity of 10^{-4} Goldblatt unit of either acid or trypsin-activated follicular fluid renin.

Immunoblot analysis of ovarian follicular fluid demonstrated that a M_r 47,000 protein cross-reacted with antisera generated against pure human renal renin (Fig. 1). Thus, follicular fluid renin had a larger molecular weight than pure renal active renin.

Supernatant from cultured granulosa cells obtained either from premenopausal ovarian tissue or from IVF follicular fluid contained extremely low quantities of active renin or prorenin after 1 week of culture (Fig. 2). In contrast, eight culture wells of theca cells obtained from follicles of three premenopausal ovaries contained 3.2 ± 7 ng·ml^{-1}·hr^{-1} of active renin and 68.3 ± 14.9 ng·ml^{-1}·hr^{-1} of prorenin per well after 1 week of culture. Differences between granulosa and theca cell cultures were confirmed by morphological assessment and measurements of 4-androstene-3,17-dione and progesterone in the medium. When observed under phase-contrast microscopy, cultures of both theca and granulosa cells appeared homogenous (Fig. 3). Granulosa cells were polygonal and contained multiple lipid droplets. Theca cells were smaller and spindle shaped. The granulosa cells produced relatively high levels of progesterone and low levels of 4-androstene-3,17-dione compared to theca cells (Fig. 2). Thus, the 4-androstene-3,17-dione/progesterone ratio was 6 for the granulosa cell medium compared to 597 for the theca cell medium. This is consistent with the known relative pattern of steroid production in ovarian granulosa and theca cells (19).

Low levels of renin and prorenin were also found when culture medium was sampled every 2–3 days for up to 24 days from granulosa cells obtained from ovarian tissue of a premenopausal patient (Fig. 4). Theca cells were removed from a 3-cm follicle in the left ovary and a 1-cm follicle in the right ovary from the same patient and also cultured for 24 days. Overall, prorenin production was markedly higher in cultured theca cells from the left ovary compared to the right ovary (Fig. 4). Peak prorenin production occurred at days 6–8 of culture. The pattern of active renin production was similar. However, active renin only represented ≈1/10th or less of the total renin produced by the theca cells. All cultures contained ≈10^6 cells per well at 1 week. No detectable active renin or prorenin was found in control medium.

Table 1. Renin activity in ovarian follicular fluid

	Renin, ng·ml^{-1}·hr^{-1}	
	Ovarian follicular fluid	Human plasma
Untreated	186 ± 20	5.7 ± 0.9
pH 3.3 dialysis	3333 ± 526	23.0 ± 4.3
pH 3.3 dialysis; neutralize and incubate 3 hr at 37°C, pH 7.5	244 ± 40	6.9 ± 1.0
pH 3.3 dialysis; neutralize and incubate 3 hr at 37°C, pH 7.5; pH 3.3 dialysis	3240 ± 216	21.0 ± 5.0
Trypsin treatment	3570 ± 410	22.5 ± 4.0

Mean ± SEM of four measurements. Trypsin treatment was performed with trypsin at 50 μg/ml for follicular fluid and at 1 mg/ml for plasma (13). This was the optimum concentration of trypsin determined to activate renin in plasma and ovarian follicular fluid.

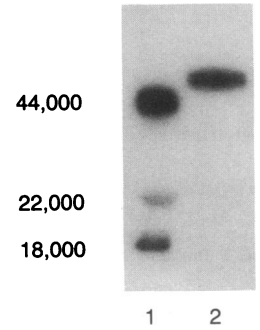

FIG. 1. Immunoblot analysis of pure active human renal renin compared to ovarian follicular fluid renin. Lane 1, pure human renal renin has a M_r of 44,000 with M_r 22,000 and 18,000 subunits. Lane 2, only a M_r 47,000 protein in ovarian follicular fluid cross-reacts with the human renin antibody.

The histologic identification of theca interna cells in human ovary was established first in hematoxylin and eosin-stained sections (Fig. 5A). A developing follicle was recognized structurally as a cystic space lined by stratified small cuboidal cells corresponding to the granulosa cell layer and containing an eccentrically placed oocyte. The layer of plump spindle-shaped cells immediately peripheral to the granulosa cell layer was identified as theca interna. The concentric compact layer of fibroblasts and collagen just outside the theca interna was identified as theca externa. These components of the developing follicle were easily recognized on corresponding hematoxylin counterstained sections used in the immunohistologic localization of renin. In a follicle containing a maturing ovum, immunostaining was most prominent in the theca interna with minimal staining occurring in the granulosa cells and theca externa (Fig. 5 C and D). This staining was absorbed out by pure human renal renin (17) and was negative in the presence of preimmune sera (Fig. 5B). Staining was generally seen in the cytoplasm of the theca cells. Four other ovaries from premenopausal women showed similar localization of renin.

DISCUSSION

This investigation demonstrates that human ovarian theca cells contain and produce renin. Both immunohistochemical staining of the developing human ovarian follicle and culture of isolated human theca and granulosa cells support this conclusion. Theca and granulosa cells were identified by light microscopy by their location and architecture. Immune staining was greatest in the area of the theca interna and was minimal in the granulosa layer.

Measurements of 4-androstene-13,17-dione and progesterone in the culture medium confirmed that the isolated cells in culture were either predominantly theca cells or granulosa cells. Morphological examination of the cells in culture indicated that the cultures were, in general, homogenous. The granulosa cell appearance was consistent with previous descriptions of cultured human granulosa cells (20). Granulosa cell medium contained little prorenin. Theca cells had a distinctly different appearance compared to granulosa cells

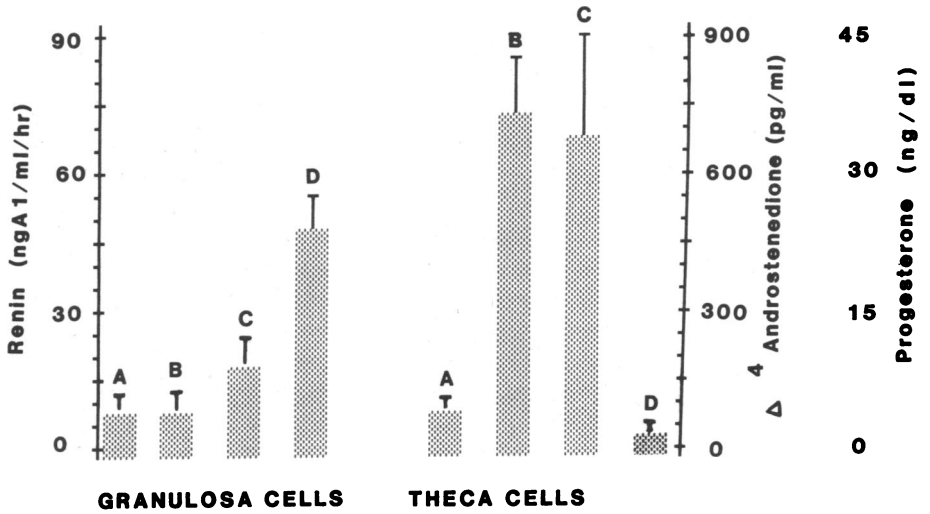

FIG. 2. Renin (A, active; B, prorenin) and steroid (C, 4-androstene-3,17-dione; D, progesterone) production in cultured human ovarian granulosa and theca cells. Mean ± SEM is represented. Theca cells produce relatively large quantities of prorenin and 4-androstene-3,17-dione, while granulosa cells produce relatively larger quantities of progesterone.

(Fig. 2). Prorenin production by human theca cells peaked after 1 week of culture. Media levels of prorenin were higher in cultures of theca cells obtained from the dominant follicle compared to theca cells obtained from a smaller follicle.

Large quantities of inactive prorenin have previously been demonstrated in ovarian follicular fluid obtained from women being prepared for IVF (14). Our studies further confirm that prorenin is the major form of renin in ovarian follicular fluid, contributing ≈95% of the total renin concentration. Ovarian follicular fluid prorenin demonstrates reversible acid activation, a specific characteristic of prorenin obtained from human kidney and plasma (14) and of recombinant prorenin (21). Exposure to low pH has been demonstrated to induce a conformational change in human prorenin that exposes the active site without hydrolysis of a peptide bond (22, 23). Prorenin then folds back to the inactive state with incubation at 37°C and neutral pH, which thus reverses enzyme activity. Immunoblot analysis indicates that follicular fluid renin has a M_r of 47,000. We report here that the molecular weight of human follicular fluid prorenin is the same as that found for recombinant prorenin and for human chorionic and amniotic fluid inactive renin (21), all of which are larger than pure active human renal renin, which does not contain the 46-amino acid prosegment (17).

Sealey and her group have also demonstrated that plasma prorenin peaks shortly after the luteinizing hormone peak during the normal menstrual cycle (24). These data strongly suggest gonadotropins may regulate theca cell renin production, as they appear to regulate Leydig cell renin production (25). Kim *et al.* (26) have recently shown that follicle-stimulating hormone and estrogen increase renin mRNA and renin activity in the rat ovary. Our data suggesting that

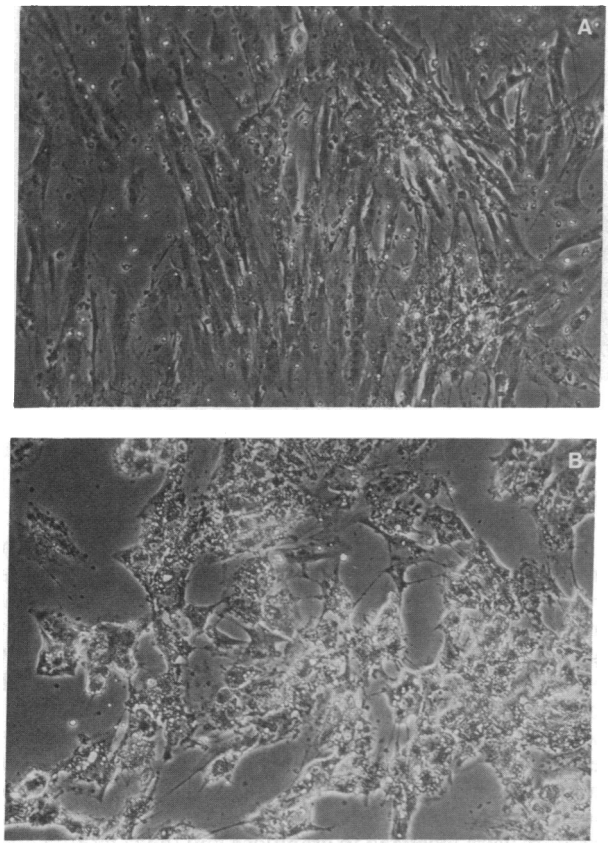

FIG. 3. Human granulosa (*A*) and theca (*B*) cells in culture after 1 week. Granulosa cells are polygonal and contain lipid droplets. Theca cells are smaller and spindle shaped.

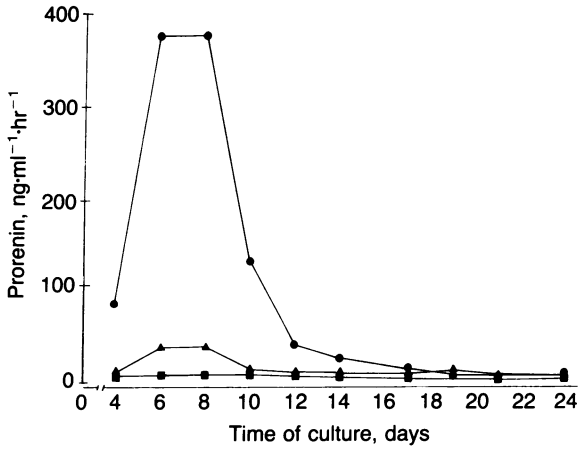

FIG. 4. Prorenin production by human granulosa (■) and theca cells in culture. All cells were taken from the same premenopausal patient during the follicular phase of her cycle. Theca cells were obtained from a 3-cm follicle in the left ovary (●) and a 1-cm follicle in the right ovary (▲). Prorenin concentrations represent the prorenin secreted into the medium per 24 hr.

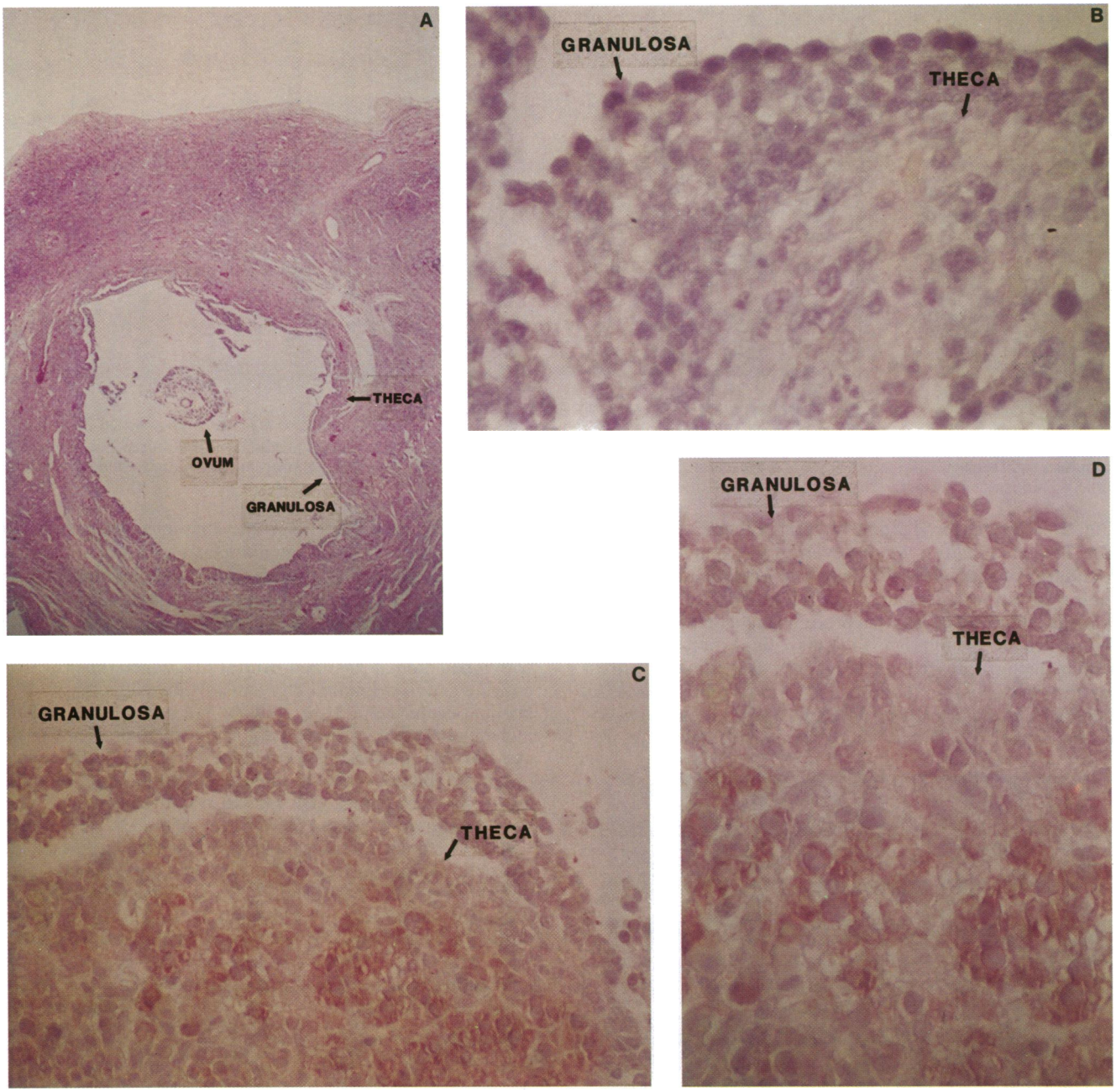

FIG. 5. (*A*) Hematoxylin and eosin-stained section of human ovary containing a maturing follicle with ovum. (×7.15.) (*B*) Hematoxylin counterstained section incubated with preimmune serum. (×140.) No evidence of positive immunoreactivity is identified. (*C*) Hematoxylin counterstained section incubated with rabbit polyclonal and anti-renin antiserum (×140.) Immunoreactivity is localized to the cytoplasm of cells within the theca with no evidence of positivity within the granulosa cell layer. (*D*) Higher magnification of *C*. (×230.)

prorenin production is increased in theca cells from the dominant follicle compared to a nondominant follicle is consistent with these observations. The dominant follicle develops the capacity to sequester follicle-stimulating hormone in the follicular fluid, which then contributes to its ability to sustain follicle estrogen biosynthesis (27).

Several possibilities could explain the lower prorenin concentrations in theca cell culture medium compared to that in follicular fluid. First, the follicular fluid was obtained from women whose ovaries were "hyper" stimulated to ovulate, while the cultured theca cells were obtained from women during the normal menstrual cycle. Second, the follicular fluid prorenin levels likely represent an accumulation of prorenin produced by theca cells over an unknown period of time. Third, the number of theca cells contributing to follicular fluid prorenin levels *in vivo* is unknown compared to the number in culture contributing to medium levels. However, estimation of the prorenin production rate per day by 10^6 theca cells from days 4–12 of culture (Fig. 4) suggests that prorenin production would approach 10^3 $ng \cdot ml^{-1} \cdot hr^{-1}$, which is a similar order of magnitude as the prorenin concentration in follicular fluid.

A complete renin–angiotensin system appears to exist in the ovary, since human follicular fluid also contains angiotensinogen and AII (14, 28). Recently, AII and AII receptors have been identified in rat ovarian tissue (29). The AII receptors were localized to granulosa and theca interna areas of some, but not all, developing follicles and were absent from granulosa areas of luteinized follicles. In addition, angiotensinogen mRNA has been found in rat ovary in

1/20th to 1/30th the levels found in the liver (30), suggesting that the ovary can also synthesize renin substrate. The finding of a complete renin–angiotensin system in the ovary is analogous to identification of the complete system in the testis. Renin and renin mRNA, AII, and converting enzyme have been identified in testicular Leydig cells (5, 31, 32).

The role of prorenin production by ovarian theca cells is unknown. Prorenin may be available to provide the relatively smaller quantities of active renin found in the ovary, which could then initiate the ultimate generation of AII. Whether prorenin has physiologic effects other than generation of AII is presently unknown. Recently, Pucell *et al.* (33) have demonstrated that AII (1 μM) stimulates estrogen production in ovarian slices from pubertal rats. This is consistent with their finding of AII receptors in granulosa cells (29). In the study of Pucell *et al.* (33), AII did not alter progesterone secretion, even though it is a major product of the granulosa cells. Thus, the effect of AII appeared specific to estrogen stimulation. 4-Androstene-3,17-dione produced by the theca cells is the major precursor of estrogen produced in the theca and granulosa cells. It is possible that the theca cell renin–angiotensin system regulates androgen production in an autocrine or paracrine fashion. Whether AII contributes to Leydig cell testosterone production is unknown. AII may have other effects in addition to steroidogenesis in the ovary. Its vasoconstricting and angiogenic properties (34) may be necessary for follicular growth.

Thus, several potential physiologic roles for ovarian renin exist. The present knowledge that human ovarian theca cells produce renin now emphasizes the direction of future studies of regulation of theca cell renin release and effects of renin and AII on theca and granulosa cell steroidogenesis. These investigations will be important to define the role of the renin–angiotensin system in human reproduction.

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