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Human Prepubertal Testicular Cells in Culture: Steroidogenic Capacity, Paracrine and Hormone Control

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The neonatal human Leydig cell undergoes a transient period of activation during the first months of life. The biological significance of this activation is unknown. Furthermore, little is known about the hormonal regulation of this biological process, even though it coincides with an elevation of LH levels in serum. In order to study the function of human prepubertal testicular culture cells, obtained during the neonatal period, a method for maintaining primary culture cells (isolated from testes collected at necropsy) in culture was developed. Within 24 h after death, testes were collected from 1–36-month-old subjects. Subjects were divided into two age groups, based on the presence or absence of fetal Leydig cells: 1–7-month-old infants (group 1) and 12–36-month-old children (group 2). Testes were digested with collagenase, and cells were seeded in multi-well dishes. Cells were grown in serum-free conditioned media supplemented with 5 mg/l vitamin C, 0.2 IU/l vitamin E and 10% fetal bovine serum for 2 days. Cells were then grown for an additional 4 days in serum-free media in the presence or absence of hLH (40 IU/l), hCG (135 IU/l), rh FSH (1.5 IU/l), rhGH (0.12 IU/l) or insulin (0.9 μmol/l). Concentrations of steroids in media were determined by RIA on day 6 of culture. In basal conditions cells of group 1 ($n = 11$) secreted more testosterone, androstendione, 17-hydroxyprogesterone, progesterone and dehydroepiandrosterone (mean ± SE: 6.76 ± 1.86, 7.37 ± 1.82, 61.9 ± 1.86, 5.75 ± 1.74 and 8.51 ± 3.23 pmol/10^6 cells/24 h, respectively) than cells of group 2 ($n = 5$) (2.95 ± 1.15, 1.50 ± 2.75, 1.44 ± 2.75, 0.78 ± 1.74 and 3.23 ± 1.32, respectively). Under hLH stimulation, cells of group 1 increased testosterone, androstendione and 17-hydroxyprogesterone secretions (to 38.2 ± 0.89, 13.5 ± 1.17 and 51.7 ± 3.23), while progesterone secretion remained unchanged (2.82 ± 1.20). Cell response to rhFSH and rhGH was similar to that of hLH. On the other hand, medium collected from cultures of cells isolated from a Sertoli cell tumor was able to stimulate testosterone secretion in subcultures of control testicular cells in a way similar to that of hCG. In conclusion, (1) these prepubertal human testicular cells can be maintained in primary culture for several days keeping their *in vivo* steroidogenic potential; (2) cells isolated from young infants can respond to hLH in culture; (3) response to rhFSH is probably mediated by a paracrine factor; (4) response to rhGH is observed in the absence of gonadotropins. Therefore, the early postnatal activation of the human testis might be under multiple pituitary hormone control; and, finally, (5) Sertoli cell tumors can secrete paracrine factors that stimulate steroidogenesis.

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INTRODUCTION

The developmental history of the human Leydig cell can be divided into three stages: fetal, early post natal and pubertal [1]. Three peaks in serum testosterone levels have been described during development: during the first half of gestation, when the male external genitalia are formed; soon after birth; and at puberty, when male sexual development takes place. The biological significance of the early post-natal phase of Leydig cell activation remains a matter of speculation.

In the early seventies, Dr Maguelone Forest and colleagues [2] reported that serum levels of testosterone peaked at 2–3 months of age in boys but not in girls. Serum androstenedione and 17-hydroxyprogesterone

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levels also increased early in life in boys [3]. Codesal *et al.* [4] determined the total number of Leydig cells per testis from the 13th week of intrauterine life to the 8th month after birth. They found a pronounced rise of fetal Leydig cells in the third month after birth, followed by a rapid decrease in such numbers. This rise in fetal Leydig cells is probably the origin of the testosterone peak detected in plasma at that age. Confirming earlier reports [5], we have also found that serum LH levels are higher in boys than in girls during the first year of life (unpublished results). Therefore, it has been concluded that an activation of the pituitary–testicular axis takes place in the human early after birth. However, gonadotropins are not the only pituitary serum hormone to be elevated postnatally. Serum growth hormone (GH) [6] and serum prolactin [7] are also high after birth, levels of GH and prolactin drop markedly after a few days, however, they remain high for several weeks thereafter.

Although no obvious physiological effect can be associated with the early postnatal activation of the testes, it has been proposed that androgen secretion after birth may have important organizational effects on the central nervous system. Moreover, it has been suggested that there may be several critical periods of sensitivity to the organizational effect of androgens [8]. Mann *et al.* [9] abolished neonatal activation of the pituitary–testicular axis with a GnRH agonist in monkeys, and they showed a reduced peripubertal rise in serum LH and testosterone. Using the same experimental model they also showed that blocking gonadotropin secretion for the first 4 months of postnatal life alters differentiation of the central nervous system centers that regulate adult sexual behaviour [10].

In order to study the endocrine and paracrine control of testicular function in sexually immature subjects, we have developed a method for maintaining in culture prepubertal human testicular cells isolated from testes collected at necropsy. Some of our results are presented in this report.

METHODS

Isolation and culture of human immature testicular cells

Testicular tissue was processed as described previously [11]. Briefly, after taking a small portion for light microscopy, testes were digested in 1.18×10^6 U/l collagenase, for 20 min at 37°C. Cells were counted with a Neubauer's chamber using Trypan blue exclusion method. Cells were seeded at a density of 1×10^6 cells per ml, 0.5 ml per well, in 24-multiwell culture dishes, resuspended in a chemically defined medium supplemented with 5 mg/l vitamin C, 0.2 IU/l vitamin E and 10% bovine fetal serum. Plates were placed at 37°C in a humidified atmosphere of 95% air, 5% CO_2. 48 h later, the medium was removed and the monolayer washed twice with Hank's solution. Fresh medium without serum was then added in triplicate (basal condition). Fresh media with hLH (NIDDK-hLH-B-1 AFP-0642 B, 40 IU/l), hCG (NIDDK-hCG 135 IU/l), rhFSH (Gonal F, Serono Laboratory, Italy, 1.5 IU/l), rhGH (Genotropin, Kabi Laboratory, Sweden, 0.12 IU/l) or insulin (Sigma Chemical Co., U.S.A., 0.9 μmol/l) were added in triplicate, in separate wells. After 48 h (day 4 of culture) media were removed and fresh media with and without hormones were added for another 48 h. On day 6 of culture, media were collected (occasionally cultures were prolongued for extra days). Media were saved and stored at −20°C for later assay of steroids. Cells were harvested after incubation with trypsin–EDTA (0.5% and 0.2%, respectively) for 3 min at 37°C, and counted as above. Steroids were determined by radioimmunoassay as described previously [11]. Aromatase activity was estimated from the mass of estradiol produced in the presence of 0.5 μmol/l testosterone.

A photomicrograph of testicular cells, on day 6 of culture, is shown in Fig. 1. Several clusters composed of cells with oval nuclei and prominent nucleoli could be seen. These epithelial cells had characteristics compatible with pre-Sertoli cells. Cellular extensions with cytoplasmic bridges radiated from these clusters.

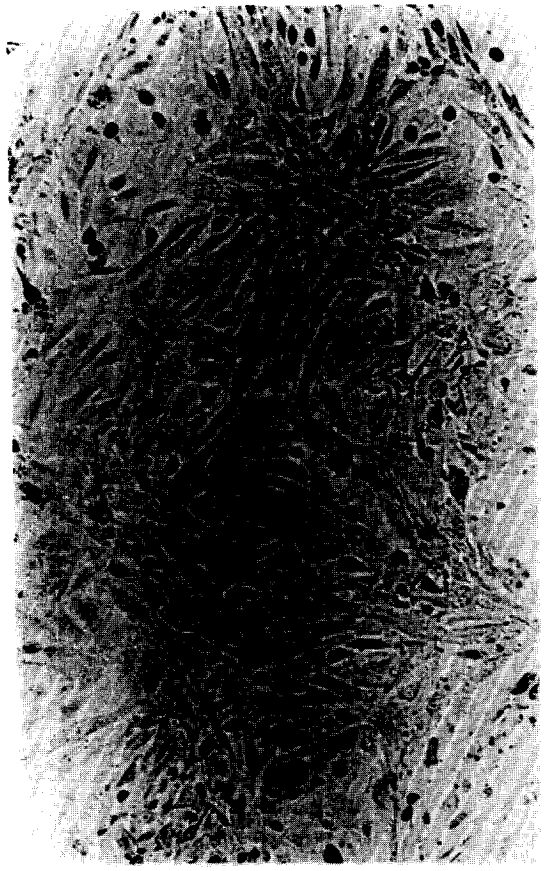

Fig. 1. Photomicrograph of a monolayer of human prepubertal testicular cells on day 6 of culture. Cells are organized in clusters of preSertoli-like cells surrounded by fibroblast-like cells showing cytoplasmic bridges.

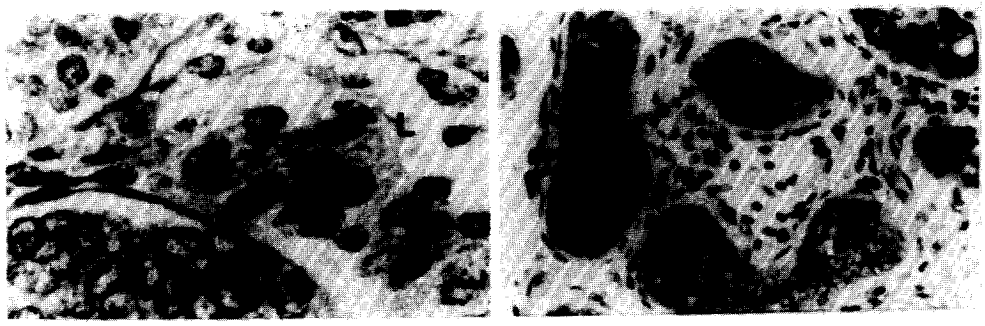

Fig. 2. Left panel: testicular histological preparation of a patient of group 1, showing fetal type Leydig cells and immature seminiferous cords. Right panel; similar preparation of a patient of group 2, in which no Leydig cells can be recognized. L, Leydig cell; T, seminiferous cord; M, mesenchymal cells.

Clinical material

Testicular cells were isolated from testes collected at necropsy of patients who died because of disorders not related to endocrine or metabolic diseases. The study was approved by the Research Committee of the Hospital de Pediatria Garrahan of Buenos Aires. Control patients were divided into 2 age groups: 0–7-month-old infants (group 1, $n = 11$) and 12–36-month-old children (group 2, $n = 5$). Cultures of testicular cells, collected at surgery from an 8-year-old boy with gynecomastia and multiple bilateral testicular nodules, were also carried out. Pathological diagnosis was a large cell calcifying Sertoli cell tumor. Two macroscopically distinct fractions of the removed testes, tumoral and extratumoral, were processed separately for cell isolation and culture [12].

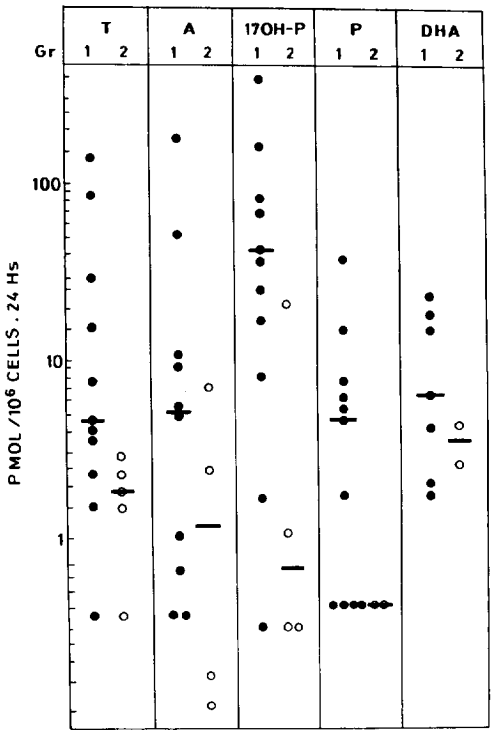

Fig. 3. Secretion of testosterone (T), androstendione (A), 17-hydroxyprogesterone (17OH-P), progesterone (P) and dehydroepiandrosterone (DHA) by testicular cells of patients of groups 1 (●) and 2 (○), under basal conditions on day 6 of culture. Horizontal lines represent means.

Testicular histology

Figure 2 shows histological preparations of representative tests of the two groups of patients. A subject of group 1 (left panel) had fetal type Leydig cells and immature seminiferous cords. No mature Leydig cells could be recognized in a patient of group 2 (right panel), but Leydig cell precursors were observed.

Statistical analysis

For statistical analysis, data were normalized by logarithmic transformation before comparison using t-tests for basal groups and paired t-tests to evaluate responses to hormone stimulation.

RESULTS

Secretion of testosterone, androstendione, 17-hydroxy-progesterone, progesterone and dehydroepiandrosterone under basal conditions

Secretion of these 5 steroids on day 6 of culture, in the two groups of patients, is shown in Fig. 3. Means ($\pm$SE) of group 1 ($n = 11$) were 6.76 ± 1.86, 7.37 ± 1.82, 61.9 ± 1.86, 5.75 ± 1.74 and 8.51 ± 3.23 pmol/10^6 cells/24 h for testosterone, androstendione, 17-hydroxyprogesterone, progesterone and dehydroepiandrosterone, respectively, while means of group 2 ($n = 5$) were 2.95 ± 1.15, 1.50 ± 2.75, 1.44 ± 2.75, 0.78 ± 1.74 and 3.23 ± 1.32, respectively. All steroids were secreted in higher amounts by cells of group 1.

Time-course of testosterone secretion under insulin stimulation during culture, in three patients of group 2

Testosterone secretion (in pmol/10^6 cells/24 h) was measured at 2, 4, 6, 8, 10 and 12 days of culture under insulin stimulation in cells isolated from 3 control patients aged 12, 18 and 36 months. As shown

in Fig. 4, the secretion of testosterone increased during culture, up to day 10. This increment was observed in the absence of gonadotropins. Judging from the patient ages, it is presumed that, *in vivo*, these testes were in the prepuberal quiescent phase of pituitary–gonadal activity. It is intriguing that under conditions of treatment with insulin, there was a progressive stimulation of steroidogenesis, even in the absence of LH.

Effect of hLH, rhFSH and rhGH on steroid secretion by cells of groups 1 and 2

Response to hLH stimulation is shown in Fig. 5. In group 1, testosterone secretion increased in 8 of 11 experiments. In group 2, it did so in 2 of 4 experiments. After incubation with the hLH, mean ($\pm$SD) testosterone secretion in group 1 (38.2 ± 2.95 pmol/10^6 cells/24 h) was significantly higher than in group 2 (2.75 ± 2.69). In group 1, androstendione secretion increased in 7 of 11 experiments. In group 2, no increase was observed in any of the 3 experiments. After treatment with hLH, mean androstendione secretion in group 1 was 13.5 ± 3.89 pmol/10^6 cells/24 h, while in 3 patients of group 2, it was 2.37 ± 2.00. In group 1, 17-hydroxyprogesterone secretion increased in 6 of 11 experiments. In group 2, it did so in 1 of 3 experiments. Incubation with hLH resulted in a mean 17-hydroxyprogesterone secretion in group 1 of 51.7 ± 10.7 pmol/10^6 cells/24 h, while in 3 patients of group 2, it was 16.1 ± 26.1. In group 1, progresterone secretion increased in only 2 of 11 experiments. In group 2, it did so in none. Mean progesterone secretion in group 1 was 2.82 ± 3.98 pmol/10^6 cells/24 h, while it was undetectable in 3 patients of group 2.

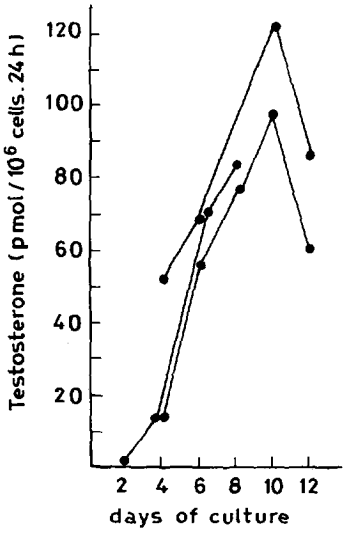

Fig. 4. Time-course of the secretion of testosterone under insulin stimulation during culture by cells of 3 patients from group 2. A gradual increase in steroidogenesis was observed up to day 10 of culture.

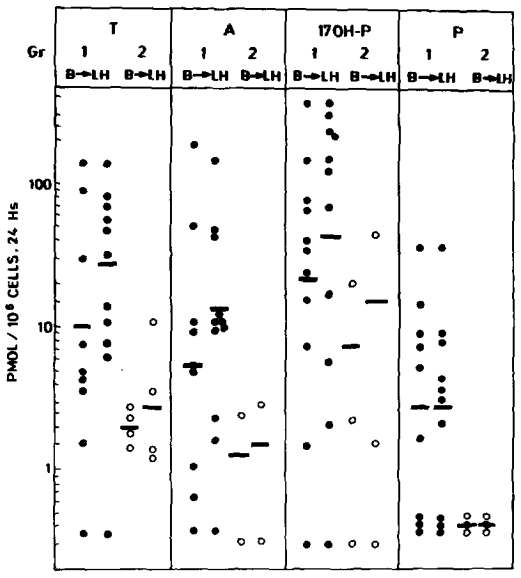

Fig. 5. Secretion of T, A, 17OH-P and P before (B) and after hLH (LH) stimulation in cells of groups 1 (●) and 2 (○). Abbreviations as in Fig. 3.

Response to rhFSH stimulation is shown in Fig. 6. In no case did treatment with rhFSH result in an increase in steroid production by cells from group 2 ($n = 2$). In group 1, testosterone secretion increased in 5 of 9 experiments. After stimulation with rhFSH, mean testosterone secretion in group 1 (28.8 ± 2.50 pmol/10^6 cells/24 h) was higher than in group 2 (1.55 ± 1.02). In group 1, androstendione secretion increased in 4 of 9 experiments. After treatment with rhFSH, mean androstendione, secretion in group 1 was 19.5 ± 2.69 pmol/10^6 cells/24 h, while in 2 patients

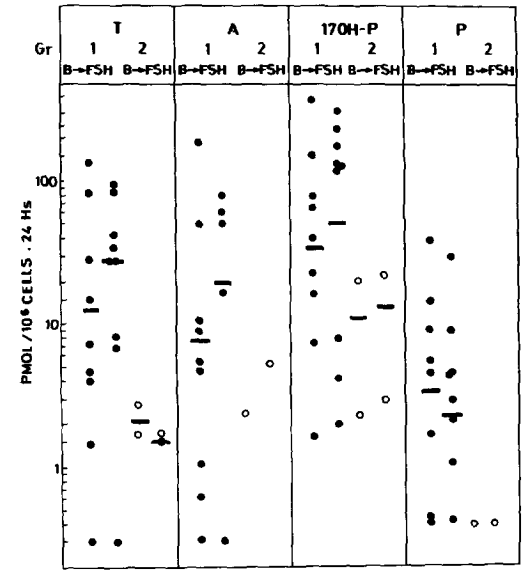

Fig. 6. Secretion of T, A, 17OH-P and P before (B) and after rhFSH (FSH) stimulation in cells of group 1 (●) and 2 (○). Abbreviations as in Fig. 3.

of group 2, it was 5.23. In group 1, 17-hydroxyprogesterone secretion increased in 5 of 9 experiments. Under rhFSH, median 17-hydroxyprogesterone secretion in group 1 was 50.1 ± 6.92 pmol/10^6 cells/24 h, while in 2 patients of group 2, it was 13.3. In group 1, progesterone secretion did not increase in any of the 11 experiments and after rhFSH treatment. Mean progesterone secretion in group 1 was 2.82 ± 3.71 pmol/10^6 cells/24 h.

Response to rhGH stimulation, is shown in Fig. 7. Again, as with treatment with rhFSH, no increase in steroid production was observed after treatment with rhGH in any patient's cells from group 2. In group 1, testosterone secretion increased in 5 of 10 experiments. Mean testosterone secretion in group 1 (19.1 ± 2.75 pmol/10^6 cells/24 h) was higher than in group 2 (2.00 ± 1.38) after incubation with rhGH. In group 1, androstenedione secretion increased in 5 of 8 experiments. Mean androstenedione secretion in group 1 (6.60 ± 4.68 pmol/10^6 cells/24 h) was higher than in group 2 (1.30 ± 1.06) after treatment with rhGH. In group 1, 17-hydroxyprogesterone secretion increased in 5 of 9 experiments. After addition of rhGH, median 17-hydroxyprogesterone secretion in group 1 was 37.1 ± 6.02 pmol/10^6 cells/24 h, while in 3 patients of group 2, it was 6.42. In group 1, progesterone secretion did not increase in any of the 11 experiments; mean progesterone secretion in group 1 was 2.45 ± 4.46 pmol/10^6 cells/24 h.

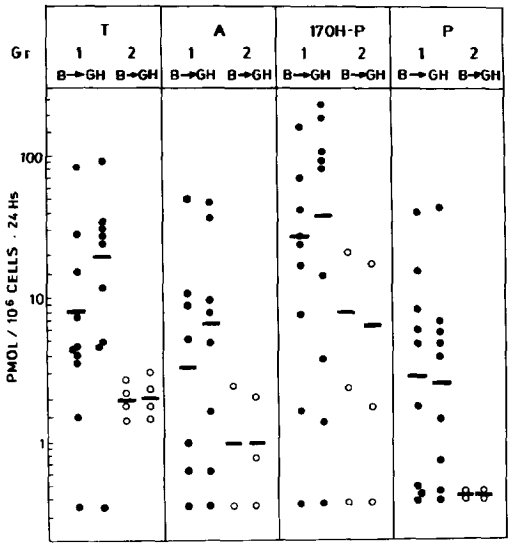

Fig. 7. Secretion of T, A, 17OH-P and P before (B) and after rhGH (EH) stimulation in cells of groups 1 (●) and 2 (○). Abbreviations as in Fig. 3.

Effect of Sertoli cell tumor secretion on testicular cells in culture

A prepubertal boy with gynecomastia underwent orchidectomy because of the presence of a testicular tumor. Two macroscopically distinct fractions of the removed testes, tumoral and extratumoral, were processed separately for cell isolation and culture. Figure 8 shows the secretion of testosterone on days 5 and 6 of culture under basal conditions, as well as under insulin and hCG stimulation, by cells obtained from the two testicular fractions. Cells from the extratumoral fraction were able to secrete testosterone, and to respond to insulin and hCG stimulation. No estradiol was detected. Cells from the tumoral fraction were not able to secrete testosterone; however, they showed high aromatase activity and this activity could be increased with the addition of insulin and FSH.

Since the extratumoral testicular parenchyma showed evidence of initiation of gonadal development, such as early signs of spermatogenesis and sparse Leydig cell differentiation in the absence of gonadotropins, the hypothesis of the existence of a stimulatory paracrine factor secreted by tumoral cells was pro-

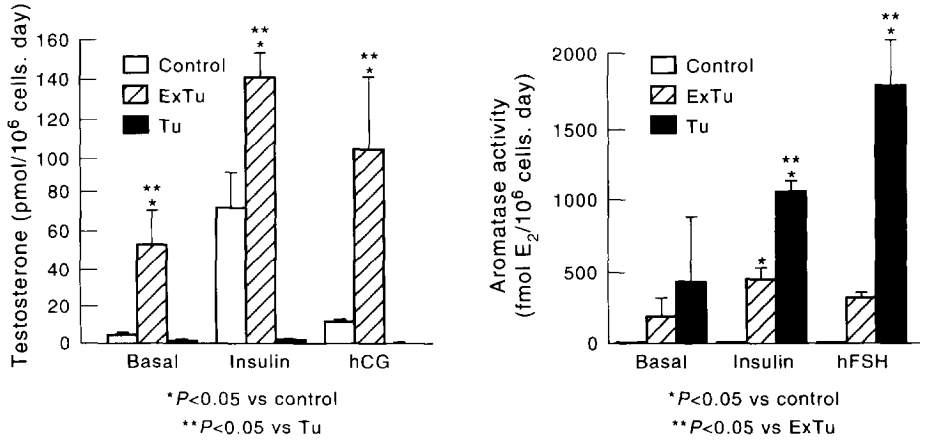

Fig. 8. Left panel: secretion of testosterone on day 6 of culture under basal conditions, as well as under insulin and hCG stimulation, by testicular cells obtained from a control patient and by tumoral and extratumoral cells from a prepubertal patient with a Sertoli cell tumor. Tumoral cells were not able to secrete testosterone. Right panel: estimation of aromatase activity in similar cell cultures. Tumoral cells showed high aromatase activity.

Table 1. *Effect of tumoral conditioned medium (TuCM) at two different dilutions (1/4 and 1.2) on testosterone secretion by a control testicular cell subculture. Comparison was made with the effect of hLH. Values in pmol/10⁶ cells/day*

	Basal	hLH	TuCM (1/4)	TuCM (1/2)
Testosterone ($\bar{X} \pm SE$)	2.05 ± 0.16	11.8 ± 1.47*	4.06 ± 0.51	12.9 ± 1.05*

*Basal vs hLH or vs TuCM: $P < 0.01$.

posed. To test this hypothesis, testicular cells from a 1-month-old control patient were maintained in culture for 6 days and subcultured in 25 ml culture flasks. Cells were allowed to grow for approx. 10 days to form a confluent monolayer. After the fifth passage on day 46 of culture, cells were plated on multiwell dishes. As shown in Table 1, these cell subcultures retained the capacity to secrete testosterone on day 5–6 of the fifth culture passage under basal conditions, as well as to respond significantly to hLH stimulation ($P < 0.01$). It was found that, after addition of the tumoral cell conditioned medium, diluted 1/4 and 1/2, mean testosterone secretion increased significantly ($P < 0.01$).

DISCUSSION

We have presented a method for maintaining in culture prepubertal human testicular cells isolated from testes collected at necropsy. These cells are able to form a monolayer in culture which is arranged in clusters of polygonal cells, resembling pre-Sertoli cells, from which extension of elongated fibroblast-like cells radiate. These cultures maintain the ability to secrete testosterone for at least 1 week. Therefore, they provide an experimental model to study the response to a variety of stimuli of testicular cells from prepubertal humans. It is also possible to compare cell function of cultures prepared from subjects at different developmental ages. In this respect, we have compared the secretion of testosterone, androstendione, 17-hydroxyprogesterone and progesterone of cells obtained from young infants (first semester of life) with that of cells obtained from 1–3-year-old children. Since cells of young infants (group 1) were more active in culture cells of children (group 2), it was concluded that these cells maintain their *in vivo* steroidogenic potential. An interesting characteristic of these cells is that they increase their testosterone secretion with days of culture, at least in the presence of insulin. Since this was observed in the absence of gonadotropins, it is possible that paracrine factors were able to stimulate steroidogenesis of Leydig cells in culture. Testosterone is considered to be the final secretory product of Leydig cells. However, since human testicular steroidogenesis follows the Δ5 pathway [13], other Δ4 steroids, such as 17-hydroxyprogesterone and progesterone, should also be considered as final products of secretion, since they might have biological effects in other tissues. In this respect, progesterone is recognized as one of the main neurosteroids [14].

Testicular cells of the young infants of group 1 were active not only under basal conditions, but they also responded to *in vitro* stimulation with hLH, rhFSH and rhGH. The ability of these cells to increase testosterone secretion after addition of hLH suggests that Leydig cells in culture kept several of their functional characteristics: presence of LH receptors, a functional intracellular messenger system and regulation of steroid enzyme transcription. In most cell cultures, androstendione and 17-hydroxyprogesterone secretion was also stimulated. By contrast, progesterone secretion did not change after hLH treatment. We speculate that a relatively higher activation of 17-hydroxylation, induced by hLH, avoided an incremental in the intracellular pregnenolone pool, necessary for progesterone synthesis.

It is well accepted that FSH receptors are only present in Sertoli cells [15]. Since these cells are not able to carry out testosterone synthesis *de novo*, it is possible that rhFSH induced the secretion of a paracrine factor by Sertoli cells which was able to stimulate testosterone secretion by Leydig cells. Interactions of human Sertoli cells with Leydig cells on steroidogenesis have been reported previously [16], and the effect of FSH on testicular steriodogenesis has also been observed in the human [17]. Similar to what we found for hLH, rhFSH also stimulated androstenedione and 17-hydroxyprogesterone production, but not progesterone secretion.

Addition to rhGH had an effect similar to hLH treatment on cells from group 1. hGH can interact with either the GH or the prolactin receptor [18]. Our data cannot differentiate these two possibilities. Since the two hormones are elevated during the first days of life [6, 7], it is possible that either one might play a role in testicular function of the newborn.

Cells of group 2 did not respond to hormone stimulation. Presumably, very few steroid producing interstitial cells were present in these cultures.

In summary, our data support the hypothesis that the early postnatal activation of the testis is under multiple pituitary hormone influence. Paracrine factors might play a role in testicular function in both healthy and disease states. We had the opportunity of study the effect of conditioned medium from cultures of a Sertoli cell tumor cell on a monolayer of subcultures of testicular cells isolated from a control subject. Conditioned medium was able to stimulate testosterone secretion in a way similar to that of hLH, suggesting that tumoral

cells secreted a stimulatory paracrine factor which had an action on Leydig cells. Similar mechanisms of action might explain some of the effects of gonadal tumors on gonadal function.

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