VIABILITY OF MEIOTIC PROPHASE SPERMATOCYTES OF RATS IS FACILITATED IN PRIMARY CULTURE OF DISPERSED TESTICULAR CELLS ON COLLAGEN GEL BY SUPPLEMENTING EPINEPHRINE OR NOREPI-NEPHRINE: EVIDENCE THAT MEIOTIC PROPHASE SPERMATOCYTES COMPLETE MEIOTIC DIVISIONS IN VITRO

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SUMMARY

Dispersed testicular cells prepared from 14-d-old rats were cultured on type 1 collagen gels using a medium composed of a 1:1 mixture of Ham's F12 medium and Leibovitz's L15 medium (F12-L15 medium) containing 10% (vol/vol) fetal bovine serum. The viability of the spermatogenic cells was facilitated by supplementing a rat adrenal extract into the medium. The effective substance(s) (the survival factor) was purified from acid extracts of adrenals by molecular sieve high performance liquid chromatography and identified as epinephrine and norepinephrine. Both epinephrine and norepinephrine promoted the survival of the spermatogenic cells with a half saturating dose of 10 ng/ml. The spermatogenic cells, which could be cultured for 2 wk on a collagen gel by supplementing with the survival factor (epinephrine or norepinephrine), were subjected to Giemsa staining and to DNA flow cytometry. The following results were obtained: a) The spermatogenic cells from 14-d-old rats did not contain spermiogenic cells (1c-cells). b) During a culture period of 2 to 7 d the ratio of meiotic prophase spermatocytes (4c-cells) to premeiotic cells (2c-cells) increased. On Day 7, more than 90% of the surviving cells were meiotic prophase spermatocytes. c) On Day 10, spermatids (1c-cells) appeared for the first time. The time of the first appearance of spermatids in the culture was consistent with that in vivo. These results suggest that both epinephrine and norepinephrine facilitated the viability of meiotic prophase spermatocytes and that a part of the meiotic prophase spermatocytes completed the meiotic divisions in the testicular cell culture.

Key words: spermatogenesis; epinephrine; norepinephrine; survival factor; meiotic division.

INTRODUCTION

Spermatogenesis is a dynamic process that includes mitotic divisions of spermatogonia, meiotic divisions of spermatocytes, and differentiation of spermiogenic cells. In vitro studies of spermatogenesis in mammals have been reported (reviews; Parvinen et al. 1986; Abé 1987). However, little clear evidence exists that primary spermatocytes complete meiotic divisions in any cell culture system. Some difficulties have occurred with dispersed spermatogenic cell cultures. However, Tres et al. (1983) reported that the viability of spermatogenic cells in vitro was facilitated by their coculture with Sertoli cells. It is generally considered that the function of Sertoli cells is regulated by follicle-stimulating hormone (FSH) and testosterone (Fritz, 1978; Ritzen et al. 1981). Several proteins produced by Sertoli cells have been described including growth factors (Feig et al. 1980; Holmes et al. 1986; Smith et al., 1987), transferrin (Skinner and Griswold 1980; Lee et al. 1986), androgen binding protein (Kierszenbaum et al. 1980; Janecki and Steinberger 1987), plasminogen activator (Lacroix et al. 1977), and proteoglycans (Skinner and Fritz 1985). However, hormones or growth factors regulating the development of specific spermatogenic cell stages and their mechanisms are not yet known. In the present study the culture conditions for spermatogenic cells of 14-d-old rats were examined, and it was found that both epinephrine and norepinephrine facilitate the viability of the meiotic prophase spermatocytes resulting in the formation of spermatids in testicular cells cultured on collagen gel.

MATERIALS AND METHODS

Preparation of dispersed testicular cells. The testes from 14-d-old Domyu strain rats were used, and dispersed testicular cells were prepared according to the method of Romrell et al. (1976) with some modifications. Briefly, 10 testes were decapsulated, and seminiferous tubules were gently expressed and then incubated in 35 ml of 0.25% collagenase in phosphate buffered saline (PBS) for 20 min at 32.5° C with occasional stirring. The seminiferous tubules were washed twice with a serum-
free medium of F12-L15 (see below), and then incubated in 35 ml of 0.25% trypsin in PBS for 15 to 20 min at 32.5°C with occasional gentle pipetting. The trypsin treatment was terminated by adding fetal bovine serum (FBS) to 10% (vol/vol). The resulting cell suspension was filtered twice through four sheets of gauze to remove cell aggregates and tissue debris, after which the cells were collected by centrifugation. The cells were suspended in 30 ml of F12-L15 medium containing 10% (vol/vol) FBS, and washed once centrifugally. Finally, the cells were suspended in the same medium containing 10% (vol/vol) FBS, and passed once through four sheets of gauze. The viability of the dispersed testicular cells, as determined by the trypan blue exclusion test, was 70 to 80%. The normal yield was 1.5 to 2.0 × 10⁷ cells/testis. Dispersed testicular cells from rats of various ages were also prepared by essentially the same method. All procedures described above were performed under sterile conditions.

Cell culture. Dispersed testicular cells obtained from 14-d-old rats were plated in Cell Wells (Corning Glass Works, Corning, NY) at a density of 10⁶ cells/well with 2 ml of medium (pH 7.25) composed of a 1:1 mixture of Ham's F12 medium and Leibovitz's L15 medium (F12-L15 medium) supplemented with 1 g/liter of sodium bicarbonate, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 15 mM N-2-hydroxyethylpiperazine-"N-2-ethanesulfonic acid (HEPES, pH 7.3) and 10% (vol/vol) FBS. As a favorable culture condition, the wells were coated with a type 1 collagen gel in most of the experiments, as described below. The cells were cultured at 32.5°C in a humidified atmosphere of 5% CO₂ in air. Various supplements were added to the medium and half (1 ml) of the medium was replaced every other day by a fresh medium.

Coating of wells with type 1 collagen gels. Four volumes of Cellmatrix type 1-A (type-1 collagen, 3 mg/ml, pH 3, Nitta Geratin Co. Ltd., Osaka, Japan) were mixed with 1 vol of a fivefold concentration of F12-L15 medium in an ice bath. A volume of 0.3 ml each of the neutralized collagen solution was added to the wells, and then allowed to stand for 20 to 30 min at 37°C. The resulting gels were washed twice with 2 ml of F12-L15 medium containing 10% (vol/vol) FBS through two 30-min incubations at 37°C, and then used for the culture.

Separation of spermatogenic cells in testicular cell culture. The spermatogenic cells in the testicular cell culture were separated from nongerminal cells, which formed a monolayer on the collagen gel or on the well as follows: After the culture medium (2 ml) was gently aspirated off, 1 ml of PBS was added to the culture. The spermatogenic cells which were present on the monolayer cells were detached from the layer by gentle pipetting. By this means, the spermatogenic cells could be almost completely detached from the monolayer (almost no cells were visible on the monolayer under microscopy) without significant contamination by the nongermlnal cells (judged by the Giemsa staining of the cells as described below). The spermatogenic cells were transferred into a 15-ml conical tube (Falcon, Oxnard, CA), collected centrifugally, and were subjected to Giemsa staining and DNA flow cytometry, as described below. This separation was only possible with cultures more than 48 h after plating because the monolayer cells were not sufficiently attached and spread during the first 24 to 48 h of culture.

Viability of the cultured spermatogenic cells was determined by the trypan blue exclusion test as follows: The upper half (about 1 ml) of the culture medium was gently aspirated off. The spermatogenic cells were detached from the monolayer cells with the remaining medium by gentle pipetting and then subjected to the trypan blue exclusion test.

After removal of the spermatogenic cells as described above, the monolayer cells remaining on the collagen gel were harvested by two treatments with 2 ml of 0.25% trypsin in PBS at 37°C. After the cells were washed centrifugally with PBS, they were subjected to Giemsa staining as described below.

Assay of cultured spermatogenic cell viability (assay of the survival factor). The viability of the spermatogenic cells in the culture was determined as follows: At each period of the culture, the number of spermatogenic cells present on the monolayer was counted in five microscopical fields (0.636 mm²/field) selected randomly at 200-fold magnification by an inverted microscope (Nikon, Diaphot-TMD, Tokyo, Japan). The values were averaged and multiplied by 315 to obtain the number of spermatogenic cells per well. This method was employed due to the presence of many dead cells, which made the

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**Fig. 1.** DNA distribution of dispersed testicular cells from 14-d-old rats. Dispersed testicular cells prepared from 14-d-old rats were subjected to the DNA flow cytometry as described in the text. **Abscissa:** channel number (fluorescence intensity). **Ordinate:** number of cells per channel. For channel standardization, DNA content at G₁ phase of rat spleen cells (diploid cells/2c cells) was set to 100 channel. Number of measured cells: 13 300.