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ENHANCED PROLIFERATION AND PROGESTERONE PRODUCTION BY PORCINE GRANULOSA CELLS CULTURED WITH PSEUDORABIES VIRUS GROWTH FACTOR (PRGF)

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The objective of this research was to study possible interactions of pseudorabies virus growth factor (PRGF) with ovarian tissue. Granulosa cells isolated from porcine ovaries were cultured as monolayers for 6 days in a control medium without PRGF and in medium supplemented with different doses of this agent. Increased population density and change towards more fibroblastic- like shape of cells cultured with 10^9 I.U PRGF was observed when compared with control culture.
The cells divided significantly faster during 6 days of culture under the influence of 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 I.U/ml of PRGF at a dose dependent manner. PRGF in a dose 10^9 I.U. added to cultured cells isolated from small and medium follicles did not influence progesterone secretion . An increase of progesterone secretion under the influence of PRGF in all investigated days of cultures was observed in cells isolated from large preovulatory follicles. The marked increase in progesterone content in PRGF treated culture in doses of 0.5×10^7, 0.5×10^8, 0.5×10^9 I.U. was observed during 4 and 6 days of culture.
The rise of progesterone content was not connected with increased number of secretory cells, but with a stimulation of production per cell. PRGF exerted no visible effect on progesterone secretion by granulosa cells from small and medium follicles cultured for 6 days.
The presented *in vitro* data provide evidence for a local action of PRGF in the follicle depending on the stage of follicular development and duration of exposure. Precise relevance of the interaction of PRGF with follicular development requires further study.

Key words: *ovarian cells, pseudorabies virus growth factor, progesterone secretion*

INTRODUCTION

Pseudorabies virus (PRV) encoded growth factor (PRGF) was detected in some cells transformed by PRV as well as in some virus infected cells cultivated

under conditions non permissive for virus replication (1). Purification of PRGF by discontinuous recycling chromatography (2) revealed that this factor consists of two biologically active components $PRGF_A$ and $PRGF_B$ both of $Mr < 1000$.

Pseudorabies virus growth factor, possessing dual, transforming and transformed phenotype repressing activity was detected in certain pseudorabies virus-infected and transformed cells (1,3). PRGF administered subcutaneously was shown to enhance growth rate and to facilitate postembryonal development of mice (4). Golais *et al.* (1) showed that PRGF exerted two effects on cells cultured *in vitro*. Non-transformed cells cultivated in the presence of PRV acquired the phenotype of transformed cells, whereas the phenotype of previously transformed cells became converted towards the normal one. Moreover, long-term PRGF treatment regimen did not result in appearance of tumors in laboratory mice.

It was shown that a crude extract from pseudorabies virus (PRV) transformed human embryonic lung (HEL) cells (H-PR-1 cell line) has a transforming activity when added to normal HEL cells. The same activity was also demonstrated in the extract of HEL cells infected with PRV. Both extracts were also able to transform mouse NIH 3T3 cells. Cultivation of transformed mouse L as well as human HeLa and H-MB-2 (melanoma) cells in the presence of both extracts resulted in the loss of their transformed phenotype and anchorage independent growth (1). The transformation frequency of Syrian hamster embryonic cells was not dependent on PRGF concentration (5).

A special interest of this study is the interaction of PRGF with endocrine systems. Described effects of PRGF (1) might be of interest in studies on tumor progression or inhibition. Before testing this agent on tumor bearing animals, the effect of PRGF on granulosa cells steroidogenesis from normal cycling pig was studied.

MATERIAL AND METHODS

Reagents

— medium 199, calf serum, trypsin were the product of the Laboratory of Sera and Vaccines, Lublin, Poland

— pseudorabies virus growth factor (PRGF) was purchased from Department of Microbiology and Virology, Comenius University, Bratislava (6)

— tritiated [1, 2, 6, 7- ^{3}H] progesterone was obtained from Radiochemical Center, Amersham, England

— antiserum to progesterone was kindly donated by Prof. Dr B. Cook, Department of Steroid Biochemistry, University of Glasgow, Scotland

— reagents for histochemical analysis such as nicotinamide adenine dinucleotide (NAD), nitro blue tetrazolium salt (NBT) and dehydroepiandrosterone (DHA) were purchased from Sigma, USA

— all reagents were of analytical grade and ethyl ether, hexane, toluene and absolute alcohol were distilled before use

Animals

Ovaries in the early, middle and late follicular phase of the estrous cycle were obtained from sows at a local slaughterhouse. The separation of granulosa cells from theca layer was performed according to Stoklosowa *et al.* (7). The cells were then washed and suspended in medium M199 supplemented with 5% of calf serum. Cell suspensions were transferred into wells of culture plates (Nunc) and incubated at 37°C in 5% CO_2 in humified air.

Experimental procedure

Experiment 1. Analysis of morphology
Sixteen cultures grown simultaneously were used for morphological analysis. Every second day cultures were terminated and observed under the microscope with Nomarski equipment.

Experiment 2. PRGF influence on cell proliferation
Cultures were carried out in triplicate for each day out of 6-day culture period and for seven doses of PRGF used (0.5×10^3, 0.5×10^4, 0.5×10^5, 0.5×10^6, 0.5×10^7, 0.5×10^8 and 0.5×10^9 I.U/ml). Every second day cultures were trypsinized with 0.25% trypsin, harvested, diluted and cells counted in a Coulter counter.

Experiment 3. PRGF influence on progesterone secretion
To show the dose response to PRGF in terms of progesterone secretion dispersed cells were incubated without (control) and with seven doses (0.5×10^3, 0.5×10^4, 0.5×10^5, 0.5×10^6, 0.5×10^7, 0.5×10^8 and 0.5×10^9 I.U/ml) of PRGF. Every second day the medium was changed and frozen for steroid analysis by radioimmunoassay.
Progesterone was detected in the culture medium by a radioimmunoassay described elsewhere (7). A highly specific antibody raised in sheep against 11α-hydroxy- progesterone hemisuccinate coupled to bovine serum albumin was used. The cross-reaction with pregnenolone was 2.9%. All other tested steroids showed less than 0.1% cross-reaction. [1,2,6,7- ^{3}H] progesterone (Radiochemical Centre, Amersham, England, sp.act. 80 Ci/mmol) was used as the tracer. The limit of sensitivity of the assay was 50 pg/ml. The coefficients of variation within and between assays were 1.5% and 2.5%, respectively.

Experiment 4. Influence of PRGF on progesterone secretion by granulosa cells isolated from follicles at different stage of development.
Follicles were classified as small (1—3mm in diameter), medium (4—6mm in diameter) and large (7—10mm in diameter). Granulosa cells isolated from follicles of all three different sizes were cultured in a control medium or with addition of 10^9 I.U. of PRGF. Concentration of PRGF used in this experiment was established based on the results of preliminary studies, described above. Every second day the medium was changed and frozen for steroid analysis by radioimmunoassay.

Statistical analysis

All data points are expressed as means ± SEM derived from at least three different replications, each in triplicate, resulting in at least nine observations. Significant differences between steroid levels in control and treated cells were compared by analysis of variance and by using Duncan's new multiple range test.

RESULTS

Morphological observations

Figs. 1b, c demonstrate increased population density and more fibroblastic like shape of cells cultured with 10^9 I.U PRGF when compared with control culture (*Figs. 1a, b*).

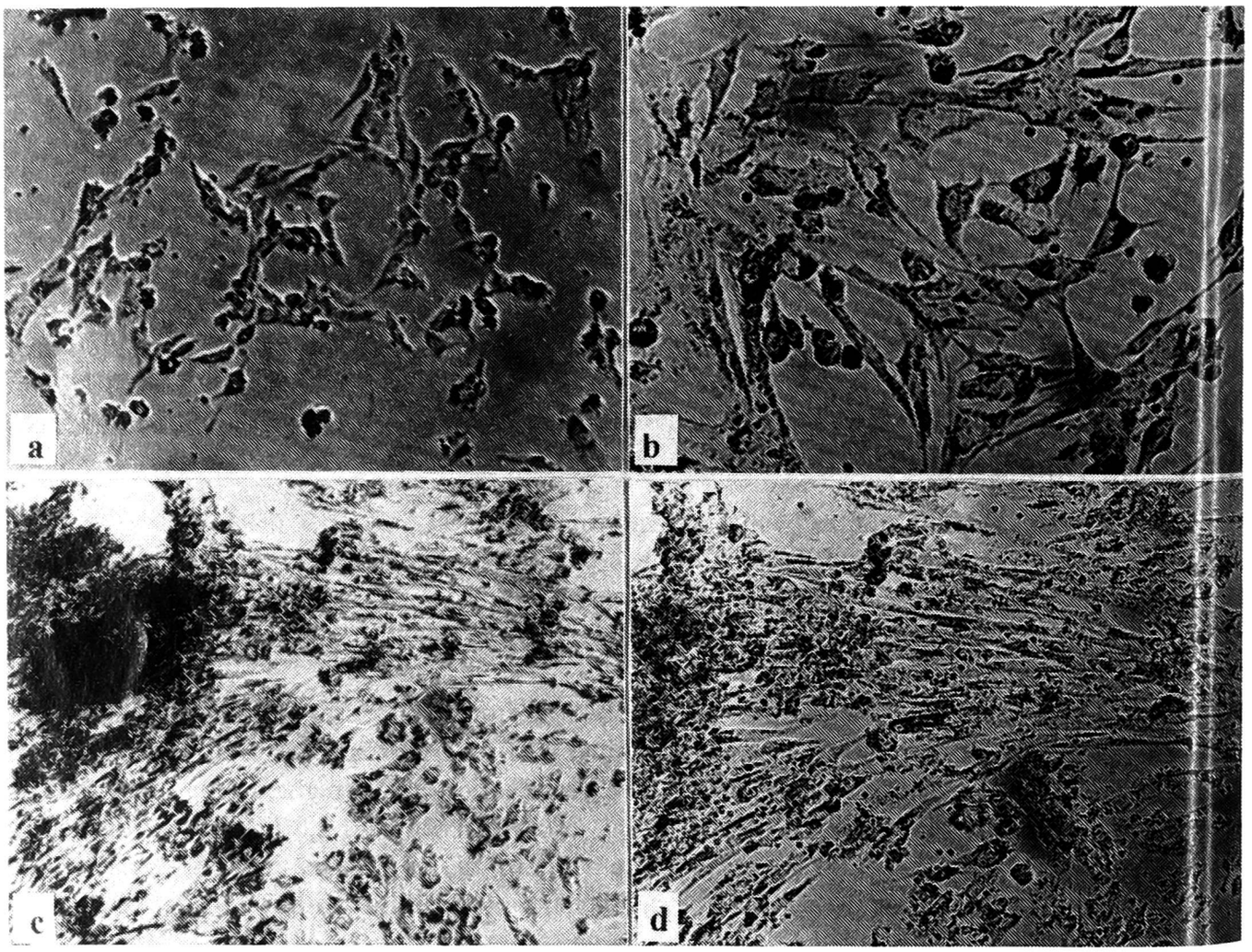

Fig. 1. Analysis of morphology. a (132 ×), b (400 ×) — control culture; c (132 ×), d (400 ×) — cells cultivated in the presence of 10^9 I.U/ml pseudorabies virus growth factor (PRGF). Nomarski equipment.

Cell multiplication

Fig. 2 represents changes in cell number during 4 and 6 days of culture. During 4 days of culture there was no statistically significant increase in the number of cells under the influence of PRGF. The cells divided significantly faster in a dose dependent manner of PRGF in 6 days of culture (3.42 ± 0.17; 4.46 ± 0.11; 4.9 ± 0.16; 6.55 ± 0.09; 7.18 ± 0.15; 7.97 ± 0.06; 10.47 ± 0.16 cells/ml, under the influence of 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 I.U/ml of PRGF vs 2.8 ± 02.1 cells/ml in control culture respectively) (*Fig. 2*).

Doses	Days of culture	
	4	6
C	1,83±0,21	2,8±0,1
$0,5\times10^{3}$	2,15±0,32	3,42±0,17
$0,5\times10^{4}$	2,23±0,21	4,46±0,11
$0,5\times10^{5}$	2,27±0,18	4,9±0,16
$0,5\times10^{6}$	3,0±0,9	6,55±0,09
$0,5\times10^{7}$	3,12±0,39	7,18±0,15
$0,5\times10^{8}$	3,17±0,29	7,97±0,06
$0,5\times10^{9}$	3,32±0,49	10,47±0,16

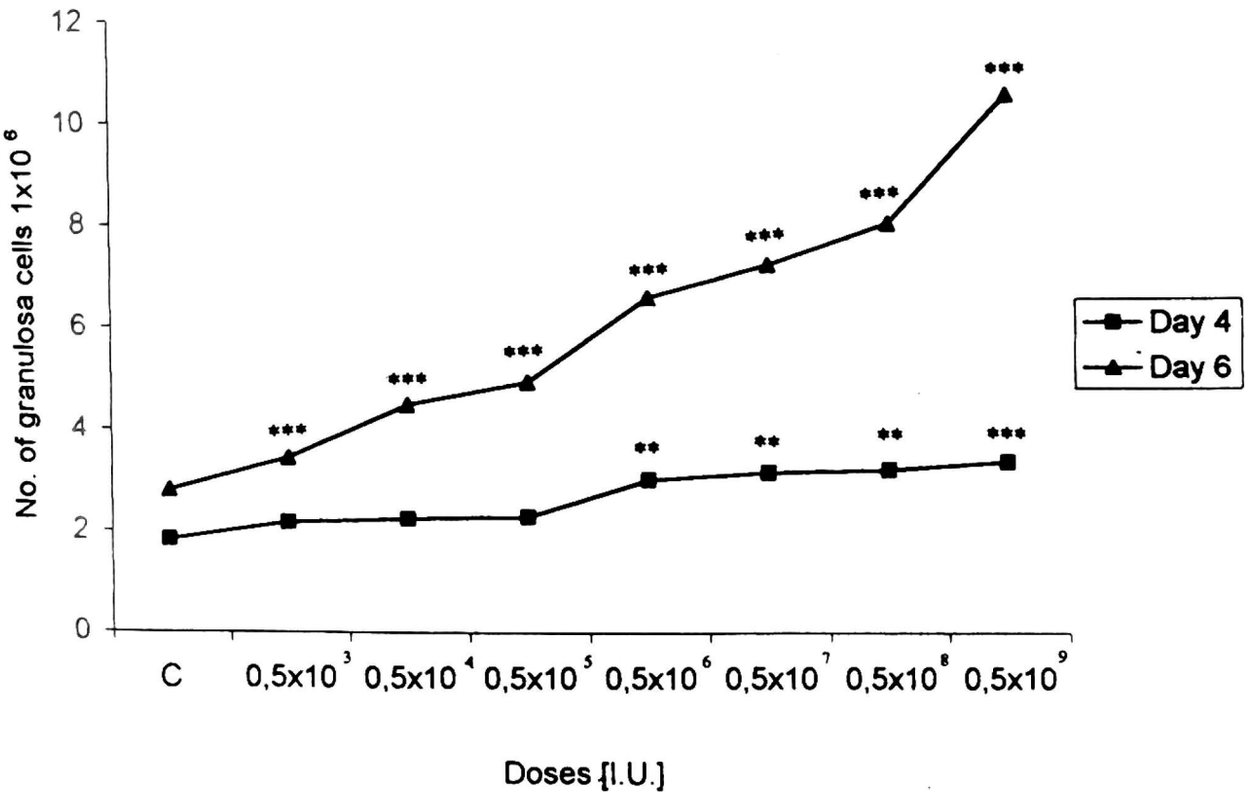

Fig. 2. Proliferation of granulosa cells with pseudorabies virus growth factor (PRGF). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Progesterone secretion

Fig. 3 represents progesterone secretion per 10^5 cells during 4 and 6 days of culture. It shows marked increase in progesterone synthesis by cells isolated from large preovulatory follicles treated with PRGF at doses of 0.5×10^7, 0.5×10^8, 0.5×10^9 I.U/ml.

The rise of progesterone content is not connected with the increase in number of secretory cells (*Fig. 2*), but with a stimulation of production per cell

(4.36, 4.40, 5.05 ng/10^5 cells under the influence of 0.5×10^7, 0.5×10^8, 0.5×10^9 PRGF respectively vs 1.91 ng/10^5 cells in control).

PRGF exerted no visible effect on progesterone secretion by granulosa cells from small and medium follicles cultured for 6 days (*Fig. 3*).

	Days of culture	
Doses	**4**	**6**
C	1,91±0,31	2,26±0,23
$0,5\times10^3$	2,32±0,33	2,32±0,23
$0,5\times10^4$	2,58±0,15	2,18±0,09
$0,5\times10^5$	2,75±0,31	1,92±0,16
$0,5\times10^6$	2,06±0,14	1,84±0,12
$0,5\times10^7$	4,36±0,55	2,2±0,27
$0,5\times10^8$	4,4±0,86	2,04±0,39
$0,5\times10^9$	5,05±0,97	1,8±0,33

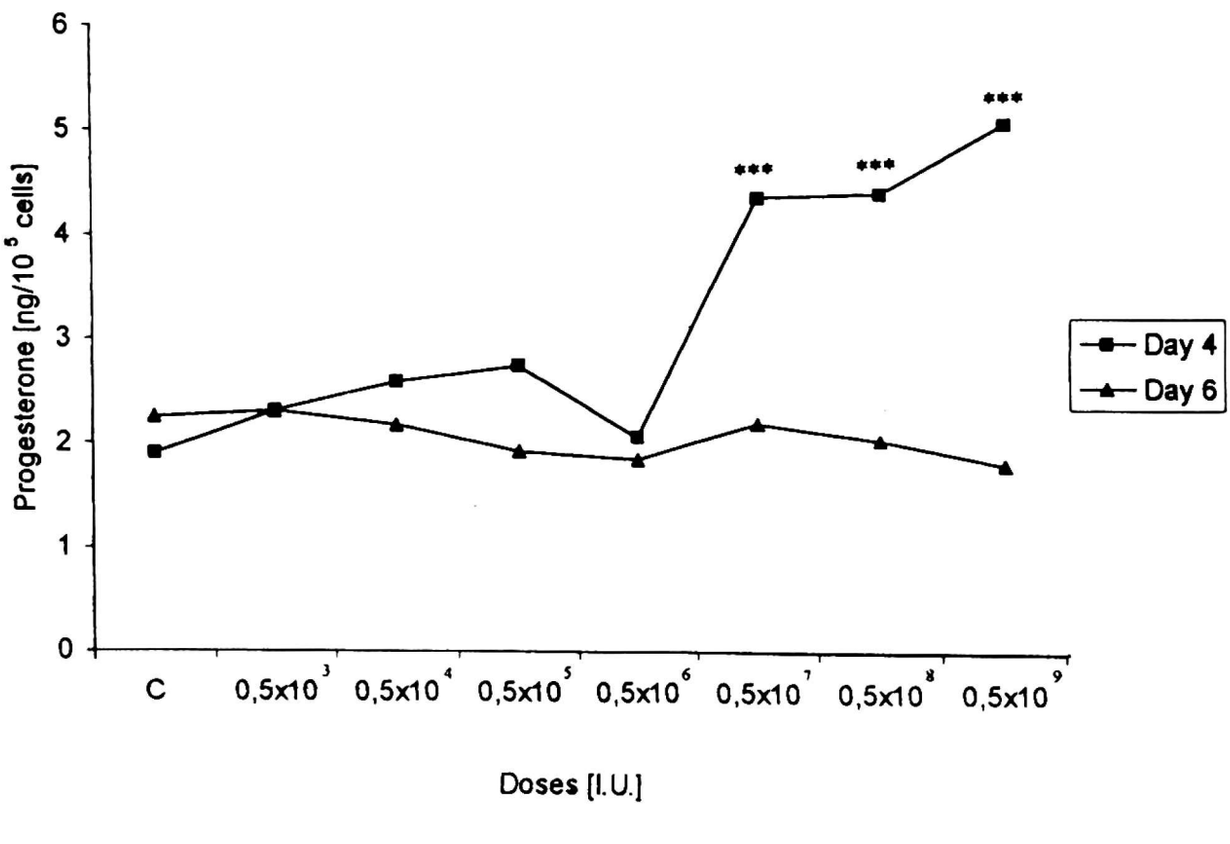

Fig. 3. Secretion of progesterone by granulosa cells isolated from large follicles (7—10 mm) cultured with increasing concentrations of pseudorabies virus growth factor (PRGF). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Dependence of progesterone secretion on follicular size

PRGF in a dose of 10^9 I.U added to cultured cells isolated from small and medium follicles did not influence progesterone secretion (*Fig. 4*). An increase

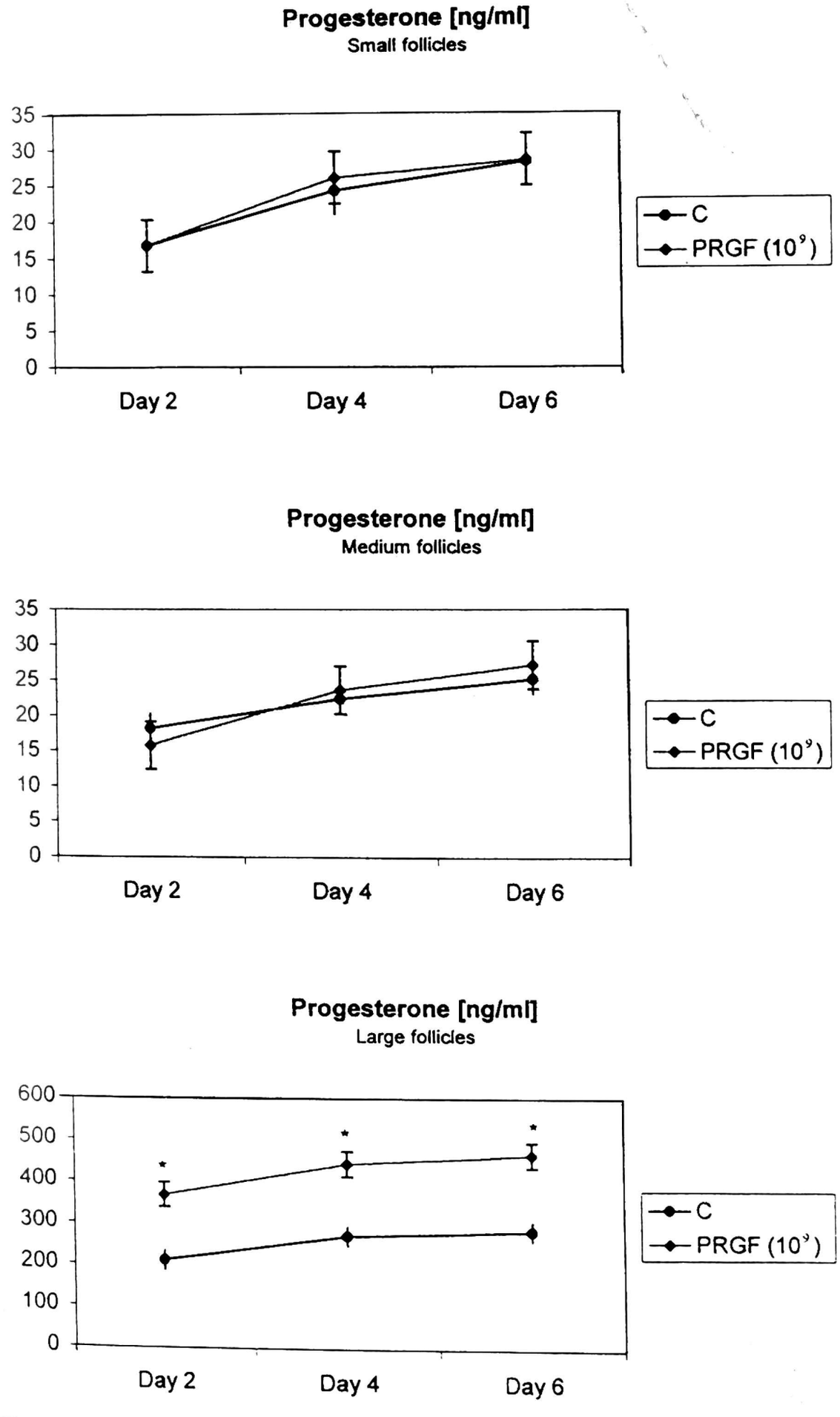

Fig. 4. Secretion of progesterone by cultured granulosa cells isolated from small (1—3 mm), medium (4—6 mm) and large (7—10 mm) follicles in the presence of 10^9 I.U/ml pseudorabies virus growth factor (PRGF). *P < 0.05, **P < 0.01, ***P < 0.001

of progesterone secretion under the influence of PRGF in all investigated days of culture was observed in cells isolated from large preovulatory follicles (367.04 ± 29.93; 443.54 ± 33.53 and 456.46 ± 35.24 ng/ml on day 2, 4 and 6 vs 209.86 ± 20.7; 269.9 ± 34.8; 283.23 ± 37.65 ng/ml on day 2,4,6 in control culture respectively).

DISCUSSION

Cultures of granulosa cells isolated from ovarian follicles represent a valuable model for the study of the role played by these cells in progesterone production and on factors which regulate their function *in vitro* and *in vivo*.

Granulosa cells isolated from large preovulatory follicles develop in a way similar to their differentiation *in vivo*, they luteinize and produce progesterone.

Growth factors related to some herpesviruses were obtained and partially characterized by Gasperik *et al.* (8). Same poxviruses have been shown to code for growth factors; however, these factors represent polypeptides homologous to cellular epidermal growth factor (EGF), or alfa transforming growth factor (TGFα) (9—11). Transforming growth factor α is implicated as a paracrine growth factor in the regulation of human granulosa cell function. TGFα is known to possess mitogenic action in the ovarian cells of several species (12, 13). Carson *et al.* (12) showed that TGFα enhanced cell proliferation and inhibited function of differentiated cells. In the presented data the cells divided significantly faster in a dose dependent manner of PRGF in 6 days of culture. On the other hand, TGFα increased basal and follicle-stimulating hormone (FSH)-stimulated progesterone production by rat (14) and porcine (15) granulosa cells.

The stimulatory action of PRGF on progesterone production by granulosa cells that we observed on day 4 was similar to that reported under the influence of TGFα. The rise of progesterone content is not connected with increased number of producing cells (*Fig. 1*), but with a stimulation of production per cell.

Taking into consideration that no tumors or visible pathological findings (as revealed by autopsy) were found in animals subjected to long-term PRGF treatment (4) these autors suggested some applications in animal husbandry. PRGF administered subcutaneously was shown to enhance the growth and to facilitate postembryonal development of mice.

The presented *in vitro* data provide evidence for a local action of PRGF in the follicle but this action, like in the case of TGFα (16) depends on the stage of follicular development and duration of exposure. Csabayova *et al.* (4) suggested PRGF as a potent novel growth stimulator. Precise relevance of the interaction of PRGF with follicular development requires further study.

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