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Role of Tumor Necrosis Factor in Preovulatory Follicles of Swine

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ABSTRACT

The effects of tumor necrosis factor (TNF) on cultured porcine granulosa cells that were obtained from preovulatory follicles were studied with regard to following parameters: 1) TNF receptor type I expression, 2) progesterone receptor and transforming growth factor β receptor type II (TβR II) as markers of luteinization, 3) proliferation, and 4) apoptosis. For comparative purposes the effects of TNF were also studied on insulin/forskolin-treated cells, as this treatment is well established to induce luteinization. Cytochemical methods followed by semiquantitative analysis were used. Our data show that TNF treatment upregulates TNF receptor type I expression in granulosa cells. TNF downregulates the expression of TβR II of insulin/forskolin-stimulated and of unstimulated cells. The progesterone receptor is also downregulated by the cytokine after insulin/forskolin-induced luteinization. Supplementation of the medium with TNF leads to increased proliferation and at the same time it induces apoptosis. Our results indicate that TNF exerts an inhibitory influence on luteinization and that TNF influences the balance between follicular growth (proliferation) and atresia (apoptosis).

apoptosis, corpus luteum function, follicular development, granulosa cells

INTRODUCTION

There is an increasing body of evidence that cytokines are involved as regulatory factors in follicular development. One of the most frequently studied cytokines in the ovary is tumor necrosis factor (TNF). It was first described as a product of activated macrophages that lysed tumor cell lines [1]. Another capacity of TNF is to elicit inflammatory responses [2]. Because ovulation has similarities to an inflammatory process [3], and TNF is produced in the ovary [4], its function there is discussed with respect to ovulation [5]. Moreover, the role of TNF in steroidogenesis, proliferation, apoptosis, and luteolysis has been studied in several species [6]. However, many of the findings are highly controversial. For instance, some authors assume a supportive function of TNF during luteinization in the pig [7], others claim a central role of TNF during luteolysis in the same species [8]. For the evaluation of possible effects of TNF on luteinization of granulosa cells (GCs) we used a culture model with GCs that were obtained from preovulatory follicles. We studied the influence of the cytokine on two markers for luteinization, the expression of the transforming growth factor β1 receptor type II (TβR II) and of the progesterone receptor (PR) by immunocytochemistry. Both have been shown to be highly related to luteinization of granulosa and theca cells in primates [9, 10]. TNF effects were additionally compared with those of insulin/forskolin, two factors that are established to initiate luteinization [11].

Another disputed point is whether TNF induces proliferation or apoptosis in the ovary. A recent study on marmoset monkeys described that steroidogenic cells in corpora lutea do proliferate [12], and furthermore it was shown that proliferation of luteal cells of different species may be influenced by several cytokines, e.g., fibroblast growth factor-2 [13], epidermal growth factor [14], and insulin-like growth factor-II [15]. On the other hand, experiments with in vitro cultures of human granulosa cells (obtained from preovulatory follicles of women undergoing in vitro fertilization) showed that TNF induces apoptosis in this cell type [16]. To this end, we investigate the influence of TNF on proliferation of GCs using the proliferation marker Ki-67. Possible apoptotic effects of TNF were examined in our cell system by detection of DNA strand breaks (TUNEL method) and translocation of phosphatidylserine (annexin V).

Functional receptors are required if TNF is to play a role in ovarian function or development. There are two major types of TNF receptors: type I (TNFR I) and type II (TNFR II). Signaling through the TNFR I is necessary for many biological functions of TNF, e.g., cytotoxicity, growth stimulation of fibroblast cells, and antiviral activity [17]. The role of TNFR II is less understood and has been shown to be more restricted to hematopoetic cells [18]. A recent study on TNFR during ovarian development confirmed the prevalence of TNFR I in rat GCs [19]. Here we asked whether TNFR I in porcine GCs is influenced by its ligand. Our study was based on immunocytochemistry and subsequent semiquantitative analysis.

MATERIALS AND METHODS

Cell Material

Porcine ovaries were obtained from the local abattoir. Granulosa cells of preovulatory follicles were harvested in accordance with the method described by Luck et al. [20]. For that, large antral follicles (>6 mm in diameter) were used [21]. Generally, follicles that had dark or cloudy fluid or thick outer membranes as indicative for atresia were discarded. These follicles were most frequent in ovaries that contained large active corpora lutea. Thus, GCs from ovaries of the late luteal and follicular phase were pooled. For each experiment at least 20 ovaries were used. Granulosa cells were scraped and then aspirated and stored in PBS (Biochrom, Berlin, Germany). The cell suspension was washed three times in PBS and cells were resuspended in culture medium (Dulbecco modified Eagle medium; Biochrom) supplemented with penicillin/streptomycin (1%; Biochrom) and FCS (2%; Biochrom). Fibroblasts were removed by preincubation in plastic culture ware at 37°C for 1 h according to a method developed by L. Pitzel (personal communication). The preincubation of the cell suspension results in a fast attachment of fibroblasts while GCs stay in suspension for a longer period. The fibroblast-free cell suspension was removed carefully and plated on laminin-coated glass tiles in plastic tissue culture ware (24-well plates, 16-mm diameter; Nunc, Wiesbaden, Germany). Laminin (2 $\mu g/cm^2$; Sigma, Deisenhofen, Germany) allowed optimal attachment of the GCs. Cells were plated at a density of 1×10^5 cells/well and were incubated overnight before the experiments were carried out.

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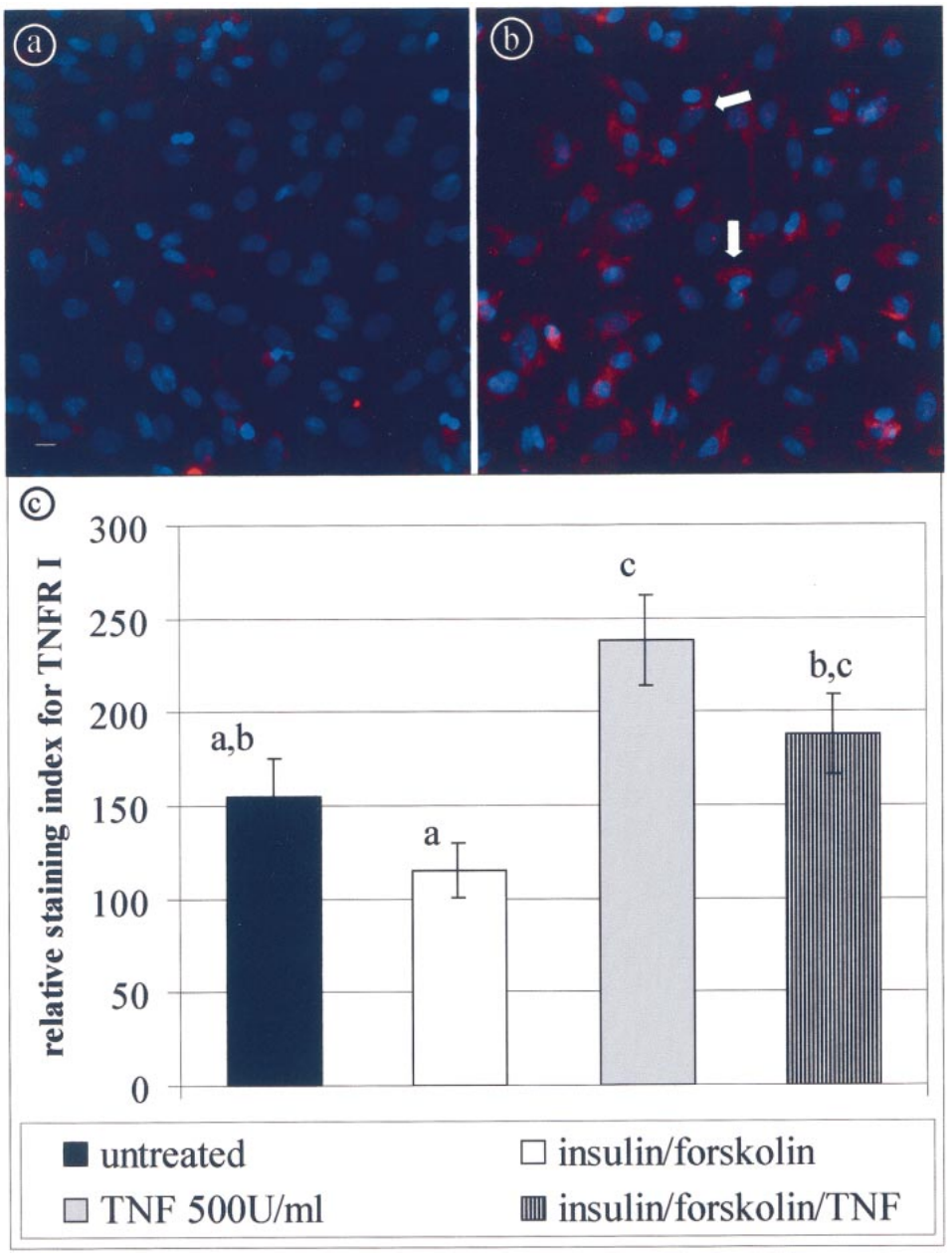

FIG. 1. Immunocytochemistry of TNFR I in porcine GCs. The GCs were cultured for 48 h without treatment (**a**) or after TNF treatment (500 U TNF/ml) (**b**). Nuclei are stained with DAPI; arrows indicate spot-like staining in the perinuclear area. Bar = 10 μm. **c**) Semiquantitative measurement of TNFR I staining after 48 h of culture. Data present means ± SEM of five experiments; different letters indicate significant differences ($P < 0.05$) between treatments.

Treatment

The cells were treated for 48 h under standardized, controlled conditions (37°C, 5% CO_2). Culture medium and additional substances were changed after 24 h. Cells were treated with insulin (50 ng/ml; Sigma), forskolin (2 μg/ml; Sigma; comparable dosages of these substances have been described to induce luteinization [22]) and TNF (recombinant human TNF; R&D Systems, Wiesbaden, Germany) at a dose as indicated for each experiment. The selection of the dosages followed the literature concerning effects of TNF on ovarian cells [6] and was tested in preliminary experiments. The doses of TNF are given in activity units per ml (U/ml).

Immunocytochemistry

After fixing of cells with 3.7% formaldehyde (in PBS; Sigma) and Triton treatment for membrane permeabilization (0.2% in PBS; Serva, Heidelberg, Germany), nonspecific binding sites were blocked with BSA (3% in PBS; Biomol, Hamburg, Germany). This was followed by incubation with the primary antibody for 1 h. The following antibodies were used: Ki-67 (1:100; Dianova, Hamburg, Germany), PR (undiluted; Coulter-Immunotech, Hamburg, Germany), and TNF-receptor type I (1:50; R&D Systems). The detection of antibody binding was achieved with Cy3-labeled secondary antibodies (1:300; Jackson ImmunoResearch Laboratories, West Grove, PA) directed against the primary antibody. 4′,6′-Diamidino-2-phenylindole (DAPI) counterstaining (Boehringer, Mannheim, Germany) of the nucleus was performed before the coverslips were applied. The examination of cells was carried out with a fluorescence microscope (Axioskop; Zeiss, Oberkochen, Germany) equipped with a video camera (Hamamatsu, Suna Yama-Cho, Japan) and a cell-imaging system (Openlap; Improvision, Coventry, England) for digital data analysis. For TβR II detection (Santa Cruz Biotechnology, Heidelberg, Germany) a commercial detection system (APAAP-Dualsystem, Dianova) was used and proceeded in accordance with the manufacturer's instructions. The specificity of all antibodies was established by using nonspecific serum. For semiquantitative analysis of immunostaining we measured the size of the stained area (expressed as the amount of pixels) and the intensity of the staining (expressed via a gray scale). A relative staining index was determined by multiplying the stained area with the intensity of staining and dividing the result by the number of examined cells. The TβR II amount was analyzed by calculating the stained area as a percentage of whole cell area; therefore parts of the different cultures with comparable numbers of cells were chosen.

Apoptosis

For examination of apoptosis the detection of DNA strand breaks (TUNEL method) and staining of translocated phosphatidylserine in the cell

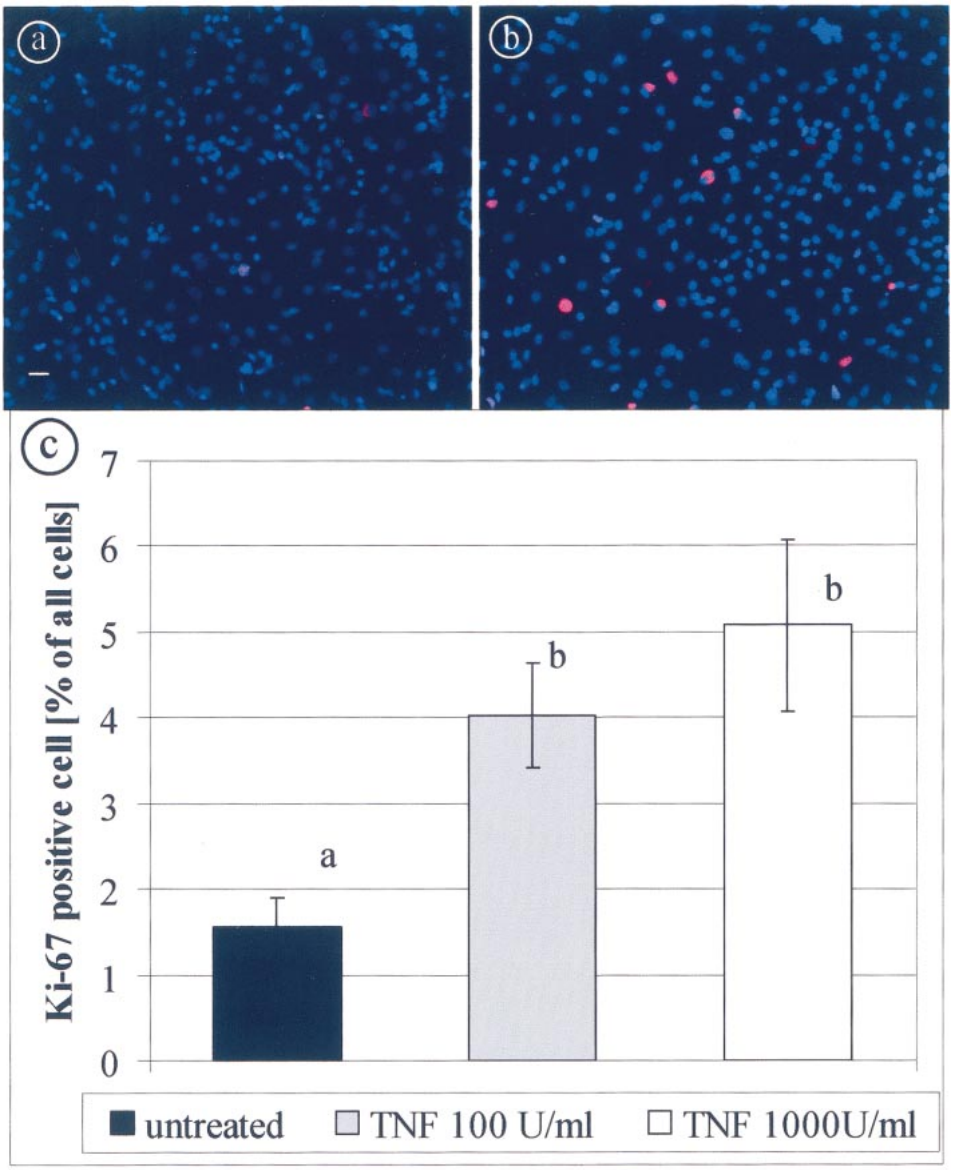

FIG. 2. Detection of proliferation with Ki-67. Compared to untreated cells (**a**), the treatment of GCs with TNF (1000 U/ml) (**b**) results in an increase in Ki-67-positive cells. Ki-67-positive cells, indicating proliferation, appear violet; Ki-67-negative cells appear blue due to DAPI staining. Bar = 50 μm. **c**) Effects of TNF on proliferation rate. Data present means ± SEM of five experiments; different letters indicate significant differences ($P < 0.01$) between treatments.

membrane (annexin V staining) were used. Porcine granulosa cells were cultured on chamber slides (Nunc) coated with laminin for 48 h under the same conditions and at the same density as described above.

TUNEL Method

Apoptosis was examined by the TUNEL method using a commercial kit (In Situ Cell Death Detection Kit POD; Boehringer). For cell counting the nuclei were counterstained with DAPI.

Annexin V Staining

Apoptotic and necrotic cells were detected after 48 h of treatment. For determination of cell death, first cultures were stained with fluorescein-labeled annexin V, a protein with high binding affinity for phosphatidylserine. For discrimination of apoptosis and necrosis, cells were subsequently stained with propidium iodide to identify necrotic cells. We used the Annexin-V-FLUOS Staining Kit (Boehringer) in accordance with the manufacturef's instructions. Examination of the cells was carried out with the fluorescence microscope (see above).

Measurements and Statistics

Each set of experiments was repeated at least five times under identical conditions; each treatment was performed in duplicate within each experiment. For analysis of the relative staining index (TNFR I) and of the stained cell area (TβR II), four fields (20× objective) out of each well were measured, each field corresponds to approximately 100 cells. For parameters that were given as ''positive as percentage of all cells'' (Ki-67, apoptosis, PR), a lower magnification (10× objective) was used. As a rule 1000 cells were examined per well. All measurements were performed blind in the sense that the investigator did not know the experimental protocol of the cultures being measured. Statistical analysis was performed on data by two-way ANOVA for repeated measures followed by a posthoc test (Fisher least significant difference), and $P < 0.05$ was considered to be significant.

RESULTS

TNF Receptor Type I

More than 90% of nontreated porcine GCs expressed the TNFR I. Cells were either evenly stained throughout or showed spotlike staining in the perinuclear area (Fig. 1b). A slight but not significant decrease of the relative staining index was observed after insulin/forskolin treatment, but the relative staining index increased by more than 50% in TNF-treated GCs (500 U/ml) compared to untreated and insulin/forskolin-treated cells (Fig. 1c). This augmentation was significant ($P < 0.001$). The combination of TNF with insulin/forskolin

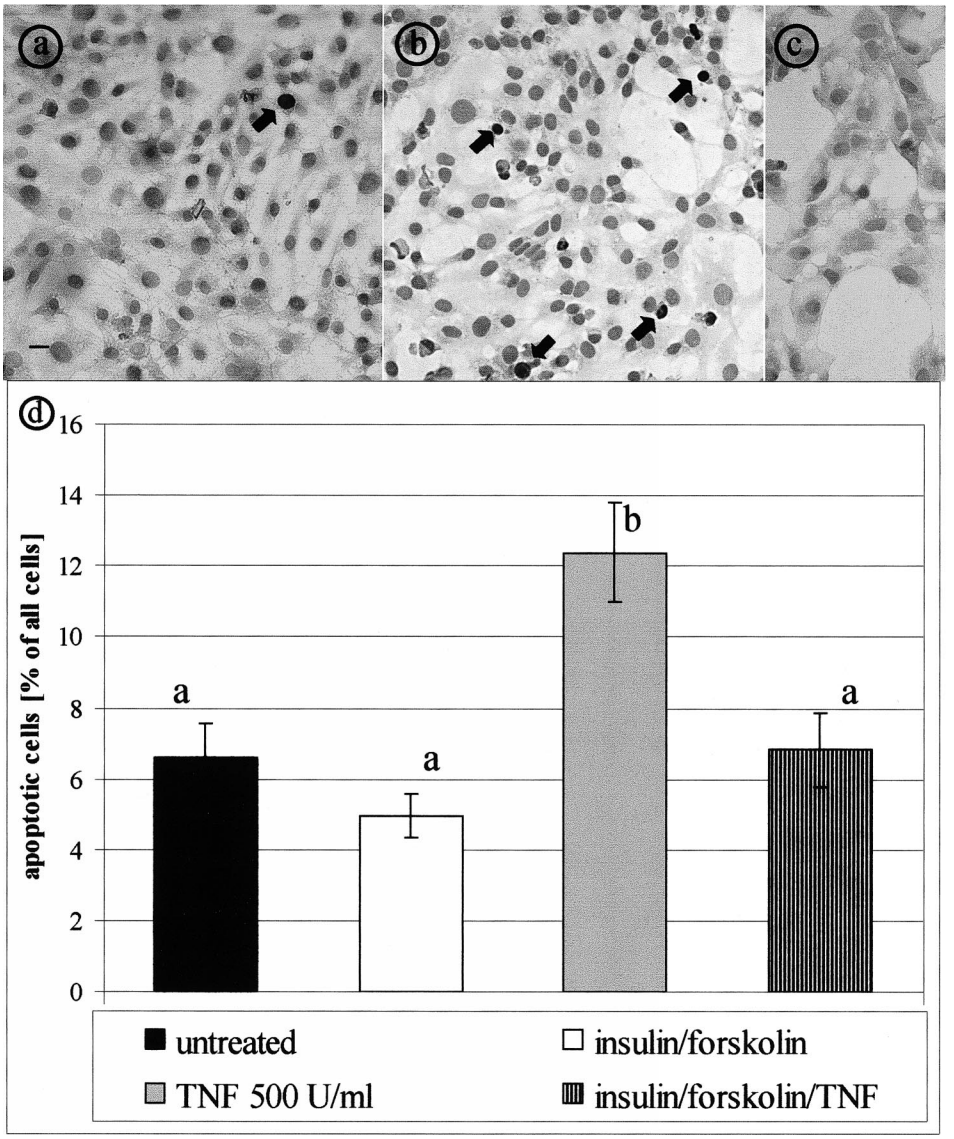

FIG. 3. Detection of apoptosis of porcine GCs by the TUNEL method. Cultures treated with TNF (500 U/ml) (**b**) showed more apoptotic cells (marked by arrows) than untreated cultures (**a**). The negative control (**c**) was free of stained cells. Bar = 10 μm. **d**) Results of cell counting. Data present means ± SEM of five experiments; different letters indicate significant differences ($P < 0.01$) between treatments.

caused a significant increase by 63% in receptor expression compared to insulin/forskolin treatment alone ($P < 0.05$).

Proliferation

The effects of TNF on proliferation of GCs in vitro was tested by treating them for 48 h with TNF at two doses (100 U/ml and 1000 U/ml). In Figure 2, a and b, Ki-67-positive cells are shown. They appear violet due to the combination of red dye (Cy3) and blue dye (DAPI). Negative cells are blue due to DAPI staining. Both TNF doses caused a significant increase ($P < 0.01$) in the amount of Ki-67-positive cells (Fig. 2c). Under control conditions (untreated cells), the proliferation rate is 1.6%, and it increased up to 4.0% (100 U) and 5.1% (1000 U) after TNF treatment. The difference between the two TNF doses was not significant.

Apoptosis

TUNEL method (Fig. 3). The application of TNF (500 U/ml) almost doubled the apoptosis rate of porcine GCs compared to untreated control (12.4% versus 6.6%). This increase of apoptotic cells was significant compared to control, insulin/forskolin and insulin/forskolin/TNF ($P < 0.01$).

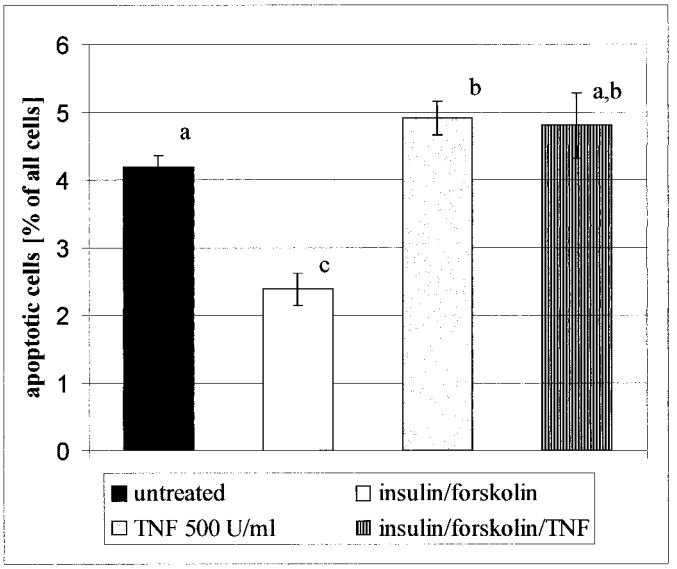

FIG. 4. Apoptosis detected by annexin V staining. Data present means ± SEM of five experiments; different letters indicate significant differences ($P < 0.05$) between treatments.

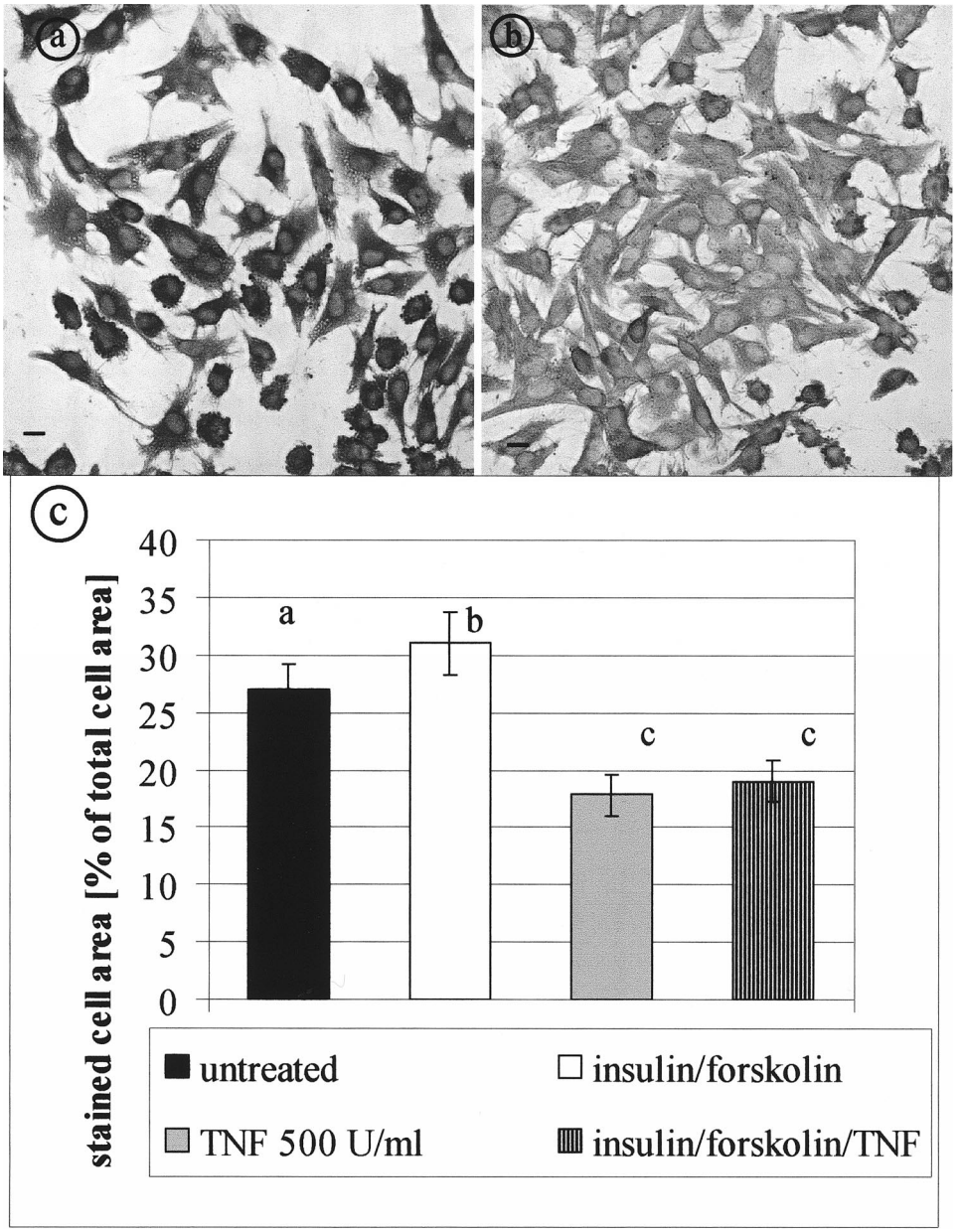

FIG. 5. Immunocytochemistry for TβR II. Insulin/forskolin treatment results in a prominent TβR II staining of porcine GCs (**a**). TNF treatment reduced the extent of stained area (**b**). Bar = 10 μm. **c**) Semiquantitative measurement of the TβR II-stained area of treated GCs. Data present means ± SEM of five experiments; different letters indicate significant differences ($P < 0.05$) between treatments.

Compared to untreated cells, the apoptosis rate of insulin/forskolin-treated cells was decreased, although the effect was not significant.

Annexin V staining (Fig. 4). In order to verify the results of the TUNEL method we also used annexin V staining to detect apoptotic cells. Again, the increase of apoptotic cells after TNF treatment compared to control and insulin/forskolin treatment was significant ($P < 0.05$), although the increase in apoptotic cells was not as high as detected by the TUNEL method. Here, the decrease in apoptosis rate after insulin/forskolin treatment was significant ($P < 0.05$) compared to untreated cells.

Transforming Growth Factor β Receptor Type II

Without treatment almost 27% of the cell area of porcine GCs was stained as the result of the immunocytochemical detection of the TβR II (Fig. 5). The treatment with insulin/forskolin induced a significant increase in the amount of stained area up to 31% ($P < 0.05$). However, the application of TNF alone or in combination with insulin/forskolin resulted in a pronounced decrease of the stained cell area to 17.8% ($P < 0.001$) and 19.1% ($P < 0.05$).

Progesterone Receptor

Immunofluorescence showed a specific distinct nuclear staining (Fig. 6). For analysis we counted the positive cells and related them to the total number of cells.

The TNF treatment resulted in a significant increase of PR-positive cells compared to untreated cells. A significant decrease ($P < 0.01$) of positive cells was found due to the combination of both treatments compared to the application of TNF or insulin/forskolin alone.

DISCUSSION

Our results support the view of TNF as a regulatory cytokine for follicular development. Several studies have shown that TNF action is mediated by intramembrane receptors in numerous cell types [18]. Because it has been shown that type I appears to be expressed typically in ova-

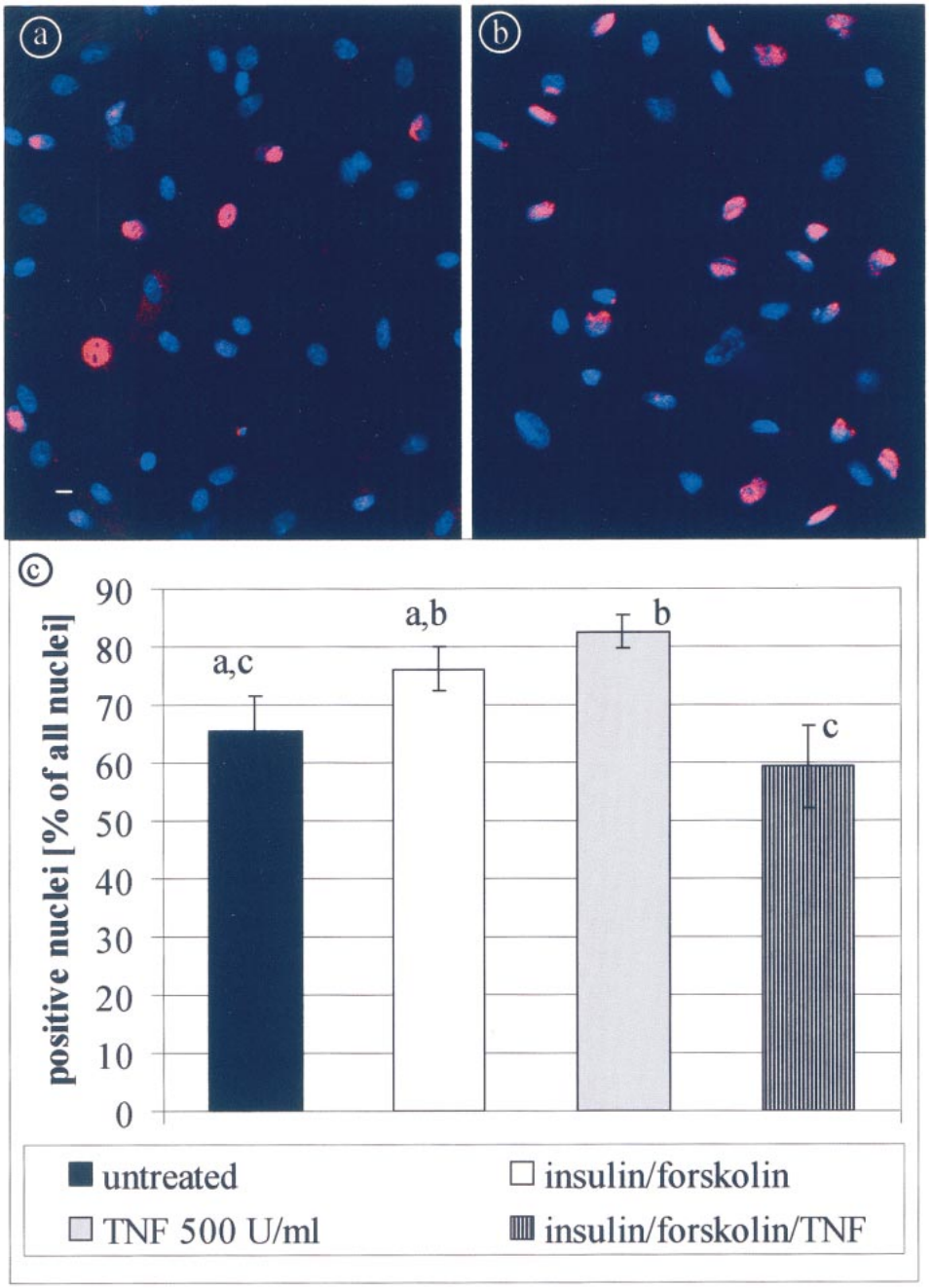

FIG. 6. Immunocytochemistry for the PR. Compared to untreated GCs (**a**) the number of positive nuclei increased after TNF treatment (**b**), due to DAPI-staining receptor-negative nuclei appear blue. Bar = 10 μm. **c**) Results of counting positive cells. Data present means ± SEM of five experiments; different letters indicate significant differences ($P < 0.01$) between treatments.

ries, we have chosen this receptor type for our study. The positive immunological signal of the TNFR I in the GCs of sows confirms that TNF effects on follicular development and differentiation are due to a receptor-mediated pathway that has already been shown in rat [19], sow [23], and mouse [24].

Little is known about the mechanisms of regulation of the TNFR I in the ovary. Veldhuis et al. [23] found an increase in binding capacity of porcine GCs after FSH/insulin treatment, suggesting an increase of receptor expression during luteinization. We induced luteinization by insulin/forskolin treatment and did not find an increase in TNFR I protein by immunocytochemistry. There are several possible reasons for this discrepancy: 1) The binding capacity of the receptor may be altered during luteinization without a change in the amount of protein. 2) Although both treatments, insulin/forskolin and FSH/insulin, are established to induce luteinization, the effect of both combinations may be different with regard to the TNFR I. 3) The GCs of both experiments may differ in their developmental stage, as Veldhuis et al. used ovaries of prepubertal pigs that may differ in LH and FSH receptor content from the GCs used in our experiment. Here, we demonstrate for the first time that TNF upregulates TNFR I expression. This is striking, insofar as in the human immunosystem and other human cell culture models the receptor has been described to be downregulated by its ligand [25, 26], which in turn points to the necessity of further studies.

Recently, a strong correlation of initial luteinization and the expression of TβR II was demonstrated in the marmoset monkey [9]. Consistently, we found that insulin/forskolin treatment at a dose that is sufficient to induce luteinization led to an increase of binding of TβR II antibodies in porcine GCs. TNF had a clearcut inhibitory effect on TβR II

expression. This effect was also seen if TNF was combined with insulin/forskolin treatment. From these results it is apparent that TNF inhibits not only induced but also basal TβR II expression, and in conclusion it demonstrates that TNF inhibits luteinization processes. With respect to TβR II expression this effect appears to be most prominent during the initial phase of luteinization. An inhibitory action of TNF on luteinization was already shown in studies on various species, where progesterone synthesis has been taken as a marker for luteinization. In several investigations TNF decreases the gonadotropin upregulated progesterone production [6]. If the expression of PR was used as a marker for luteinization, the inhibitory action of TNF on luteinization was not seen in our study when TNF was given alone in porcine GCs. However, when the medium was additionally supplemented with insulin/forskolin the inhibitory effect again became evident.

Moreover, our findings demonstrate for the first time that TNF-induced proliferation parallels TNF-induced apoptosis in the same cell system. Both TNF effects on apoptosis or on proliferation have been described in the literature. TNF is able to induce apoptosis in mouse GCs [27]. Recently, Kaipia et al. [28] detected an increase of apoptosis in antral follicles of the rat stimulated with TNF and therefore interpreted TNF as a potential factor of follicular atresia. In contrast to these data Fukuoka et al. [29] demonstrated the supportive effect of TNF on [^{3}H]thymidine incorporation of porcine GCs in vitro. Yan et al. [30] showed that TNF increased the human granulosa-luteal cell number in an in vitro study. Because in all of these studies either proliferation or apoptosis was examined, the adverse-appearing action (proliferation versus apoptosis) of TNF could not be described. The coexistence of effects of TNF on proliferation and apoptosis we found could be due to differential expression of TNFR I and II. This was shown with respect to proliferation in human [31] and in mice [32] and for apoptosis in tumor cell lines by Wong and Goedell [33]. Another possible explanation for the coexistence of effects of TNF on proliferation and apoptosis could be the heterogeneity of GCs in preovulatory follicles. This heterogeneity of GCs has already been shown with regard to proliferation, steroidogenesis, and gonadotropin receptors not only in sow [34, 35] but in various mammals [36–38].

In conclusion we demonstrated that in GC cultures porcine TNF has an inhibitory effect on luteinization and influences the balance between apoptosis and proliferation. The underlying mechanisms need further investigation.

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