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Expression of TNF-α mRNA, but not of TNF-α receptors mRNA, is detected in single murine oocyte and decreases during oocyte meiotic maturation: single-cell RT-PCR data

Background: The present study was conducted to investigate the expression of TNF-α and its receptors (types I and II) in both oocytes with germinal vesicle and the first polar body in mice.
Methods: Oocytes with intact germinal vesicle were isolated from mouse ovaries and subjected to in vitro maturation to obtain oocytes forming the first polar body. A reverse transcription polymerase chain reaction (RT-PCR) was used to examine the expression of TNF-α and its receptors at mRNA level.
Results: mRNA TNF-α was expressed in single oocytes and its level was decreasing during transition from germinal vesicle to the first polar body stage. At the same time the expression of TNF-receptors was not observed in single oocyte.
Conclusions: These data are the important link in understanding of the molecular mechanisms regulating oocyte maturation as well as follicle development.
Key words: tumor necrosis factor α, tumor necrosis factor receptors, oocytes, oocyte meiotic maturation

BACKGROUND

Understanding of the molecular mechanisms underlying oogenesis is quite important for both reproductive biology and regenerative medicine. Deciphering the complex series of regulatory events that occur during early oocyte development depends partly on the ability to accurately define gene expression pattern for certain stages of gamete development. Prior to ovulation, the metabolism of the oocyte is characterized by active gene expression. Subsequent to fertilization, a complex series of gene regulatory events occur that result in fundamental alterations in nuclear transcription [1, 24]. However, the interplay between the factors mediating development is not yet understood, precluding the elaboration of precise regulatory pathways. Gaining insight into how early developmental processes are controlled and mediated will require specific information regarding molecular events during this period. Some authors have detected expression of certain genes in oocytes [3, 5, 10, 15, 20, 25, 26]. However the technique of gene expression definition in single cells practically was not used. It is connected with a number of methodical problems particularly extraction of RNA in single cells. The scarcity of the biological material derived from single cell (oocyte is about 100 micrometers in diameter) and related to low quantity of available cells (only a few to tens of oocytes from each ovulation in mice) has hampered the molecular analysis of oocytes. Classical techniques of RNA analysis such as C_0t (the product of nucleic acid concentration and time) value assays [2], Northern blotting [22] and dot- or slot-blots [23] lacked the sensitivity to detect mRNA in single cells. Due to its unprecedented sensitivity, the reverse transcription polymerase chain reaction (RT–PCR) allows the detection of low quantity mRNA in

individual cells [16]. Several studies have been performed on single oocytes [4, 7, 8, 9, 13]. But these works did not relate to cytokines which play an essential role in oogenesis and folliculogenesis.

Tumor necrosis factor-α (TNF-α) is the cytokine produced not only by various cells of the immune system, but also by various cells in the reproductive system. It was demonstrated that oocytes are an important source of TNF-α and that the onset of oocytic TNF-α expression occurs around birth. TNF-receptors are localized on oocytes, granulosa cells and interstitial cells allowing for the possibility of autocrine or paracrine actions of TNF-α [11]. Several studies have shown that TNF-α plays an important role in follicular and luteal development, ovulation, and modulation of theca and granulosa steroidogenesis [19, 21]. Although the presence, modulation, and possible role of TNF-α has been extensively explored in ovarian function, the expression of TNF-α and its receptors has not been investigated in the murine single oocytes on two stages of oocyte development (germinal vesicle-intact oocytes and oocytes forming the first polar body). Thus, we first examined the expression of TNF-α and its receptors (types I and II) in both oocytes with germinal vesicle and the first polar body in mice. Obtained data indicated that mRNA TNF-α was only expressed in single oocyte and its level decreased during transition from germinal vesicle to the first polar body stage. That is the important link in understanding of the molecular mechanisms regulating oocyte maturation as well as follicle development.

METHODS

Animals and cells

Experiments were carried out on mature CBA female mice (18-20 g) in accordance with the International Principles of the European Convention concerning the protection of vertebrates. Mice were kept on a 12-12 hour light-dark cycle with free access to food and tap water.

Follicles were separated from ovaries and then cumulus-oocyte cellular complexes were extracted mechanically. Oocytes were mechanically denuded from cumulus cells by repeated pipetting [5] with flame-drawn glass pipette tips, whose inner diameters were slightly larger than oocytes' diameters [18]. Oocytes were washed three times with maturation medium. Atretic oocytes, as determined by their granulated appearance, were separated from healthy oocytes [12]. In order to obtain oocytes with the first polar body oocytes were cultured for 20 hours at 37°C in DMEM ("Sigma", USA), supplemented with 5% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml); ("Sigma", USA). Thus in experiments were used oocytes with intact germinal vesicle just after collection from their follicles and oocytes forming the first polar body after 20 hours of culture.

RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was obtained from fully grown, germinal vesicle-intact single oocytes and oocytes forming the first polar body using the mRNA extraction kit (Invitrogen, USA). Isolation of total RNA from oocyte before reverse transcription (RT) was absolutely necessary because RNA in oocyte formed complexes with specific message ribonucleoprotein.

First-strand cDNA synthesis was carried out by incubating of total mRNA from single oocyte with 0.2 μg/ml Random Hexamer primer (Fermentas, Lithuania) at 70°C for 1 min. A reverse transcription reaction was then prepared with the above mRNA/primer mix in 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 40 mM dithiothreitol (DTT), 1 mM of each dNTPs (Fermentas, Lithuania), 40 U of RNase inhibitor RiboLock (Fermentas, Lithuania), 20 U M-MuLV Reverse Trascriptase (Fermentas, Lithuania). The samples were incubated at 37°C for 2 h.

First-strand cDNA of single oocytes obtained after RT was used for carrying out of two rounds of polymerase chain reaction (PCR) with application of specific primers for TNF-α, its receptors and house keeping gene GAPDH (tabl). Reaction mixture for amplification was containing 25 mM $MgCl_2$, 0,5 U Taq DNA polymerase, 200 μM of each dNTP, 5 pM each of primers (or 20 pM at carrying out of the second round of amplification) and deionized water to receive total reaction volume of 25 μl. At carrying out of the second round of PCR were used the same components of reaction mixture with addition in each test-tube of 7 μl product from the first round of PCR. The first round of amplification consisted of 25 cycles and it was carried out with the following conditions: denaturation at 94 °C for 20 sec, annealing at 54 °C for 60 sec and then elongation at 72°C for 60 sec. Thermophrofile for the second round was: 94°C for 40 sec, 55 °C for 60 sec and 72°C for 60 sec repeated for 45 cycles for all pair of primers. PCR was performed in termocycler GeneAmp System 2700 ("Applied Biosystems", USA). Each experiment was repeated nine times. The resulting PCR products were then assessed by electrophoresis in 2.5% agarose gel containing ethidium bromide. Visualisation and luminance estimate were performed with application of transilluminator and software ViTran ("Biokom", Russia).

The data were evaluated statistically using Student's t-test for pairwise comparisons. Values were considered significantly different if $*P < 0.05$.

List of PCR primers used for experiments and PCR product size

Gene	Sequence of primers (forward, reverse)	Product size/bp
TNF-α	forward 5'- ATG AGC ACA GAA AGC ATG ATC -3'reverse 5'- GTC TGG GCC ATA GAA CTG AT -3'	230
TNFR-I	forward 5'-CTG CTG TCA CTG GTG CTC CTG-3'reverse 5'-CAC ACA CCG TGT CCT TGT CAG-3'	349
TNFR-II	forward 5'-GCA AGC ACA GAT GCA GTC TG-3'reverse 5'-GGT CAG AGC TGC TAC AGA CG-3'	597
GAPDH	forward5'-GGG TGT GAA CCA CGA GAA ATA TGA-3'reverse 5'-AGC ACC AGT GGA TGC AGG GAT GAT-3'	240

RESULTS

To detect expression of TNF-α gene at mRNA level we developed an amplification scheme to generate mRNA from single oocytes separated from preovulatory follicles. In each of nine experiments, RT-PCR amplification of RNA obtained from oocytes with germinal vesicle yielded a band 230 bp in size. Amplification of RNA obtained from oocytes forming the first polar body also produced identical results (Figure). Figure shows the results of TNF-α mRNA expression in oocytes.

The results of analysis of PCR products showed that intensity of the band corresponding to TNF-α mRNA expression in oocytes at the stage of germinal vesicle was significantly higher than in oocytes at the stage of the first polar body. In two of nine samples of oocytes with the first polar body TNF-α mRNA expression was undetected.

Expression of mRNA for TNF receptors in single oocytes was not detected in our experiments.

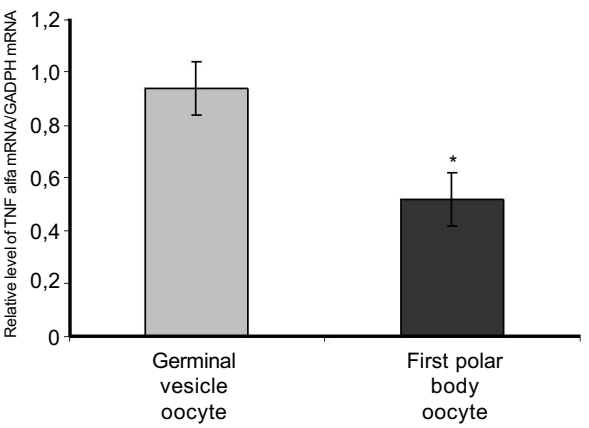
DISCUSSION

The aim of this investigation was to develop the method of gene expression detection in single murine oocytes and to identify the expression of TNF-α and its receptor systems within oocyte at two development stages (at the stage of germinal vesicle and the first polar body), that would provide important clues for understanding regulation of this cytokine and perhaps its potential function in ovary.

Our results indicate that TNF-α is expressed at the mRNA level in single murine

oocytes at both stages of their development. TNF-α mRNA expression in oocytes at the stage of germinal vesicle was significantly higher than in oocytes at the stage of the first polar body.

TNF-α expression decrease in the first polar body oocytes can be explained by sufficient quantity of the synthesised protein due to higher expression of its gene at the stage of germinal vesicle. However it is only hypothesis as we measured cytokine mRNA and not its protein, but transcription and translation of cytokines are independently regulated, therefore mRNA detection might not mirror cytokine production. We suggest also that the difference in TNF-α gene expression between two stages of oocyte development means that TNF-α has paracrine action on meiotic resumption that requires further research. At the same time our early results have indicated that TNF-α in high concentrations (relevant for inflammatory processes) exerts an inhibitory influence on meiotic maturation both in denuded oocytes and in cumulus oocyte complex [17].



Expression of TNF-α mRNA in single murine oocytes at different stages of meiotic maturation. A. Electrophoresis of PCR products of GAPDH gene: lanes 1-5 – oocytes with germinal vesicle; lanes 6-10 – oocytes with the first polar body. B. Electrophoresis of PCR products of TNF-α gene: lanes 1-5 – oocytes with germinal vesicle; lanes 6-10 – oocytes with the first polar body. C. Relative level of TNF-α mRNA / GAPDH mRNA expression in single murine oocytes

We have not detected the expression of TNF-receptors in single oocytes at the mRNA level. At the same time other scientists detected TNFR-II expression in human oocytes by RT-PCR, but not in single cells [14] and expression of both TNF-receptors in murine oocytes by immunohistochemistry [6]. In our case the absence of TNF-receptor gene expression probably can be explained: 1) by using of different research methods; 2) by species features; 3) by various stages of oocyte development (in our studies we have used oocytes obtained from large preovulatory follicles); 4) by extremely small quantity of mRNA, which is generated as a result of gene activation. The lack of TNF-receptor gene expression results in reduced influence on oocyte during oogenesis. It has been observed that TNF-induced death of oocytes requires TNFR-II. This was demonstrated by the fact that TNF-α did not kill oocytes in TNFR-II $^{-/-}$ ovaries [6]. It is possible that TNF-α is produced by oocyte for activation of TNF-receptors on other cells (cumulus, granulosa), but not for influence on oocyte directly or TNF-α action on oocytes is realized by activation of other TNF-receptors.

CONCLUSION

Thus our results indicate that TNF-α is expressed at the mRNA level in single murine oocytes at both stages of their development. TNF-α mRNA expression in oocytes at the stage of germinal vesicle was significantly higher than in oocytes at the stage of the first polar body. We suggest that the difference in TNF-α mRNA expression between two stages of oocyte development means that TNF-α could have effect on meiotic resumption (for example, through paracrine interactions between granulose cells and oocyte), but this assumption needs additional studies.

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АНАЛІЗ ЕКСПРЕСІЇ мРНК ФАКТОРА НЕКРОЗУ ПУХЛИН α ТА ЙОГО РЕЦЕПТОРІВ У ПООДИНОКИХ ООЦИТАХ МИШЕЙ ПІД ЧАС МЕЙОТИЧНОГО ДОЗРІВАННЯ: РЕЗУЛЬТАТИ ПОЛІМЕРАЗНОЇ ЛАНЦЮГОВОЇ РЕАКЦІЇ

Досліджено експресію мРНК фактора некрозу пухлин α (ФНП-α) та його рецепторів I і II типу у поодиноких ооцитах на стадіях зародкового пухирця та метафази II (формування першого полярного тільця) у мишей лінії СВА методом полімеразної ланцюгової реакції. Виявлено експресію мРНК ФНП-α, але не його рецепторів, на обох стадіях відновлення мейозу, при цьому в ооцитах із зародковим пухирцем її рівень був вірогідно вищим, ніж у клітинах з першим полярним тільцем, що вказує на можливу роль ФНП-α у мейотичному дозріванні. Отримані результати є важливими для розуміння молекулярних механізмів, які регулюють оогенез і фолікулогенез.

Ключові слова: фактор некрозу пухлин α, рецептори фактора некрозу пухлин α, ооцити, мейотичне дозрівання ооцитів.

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АНАЛИЗ ЭКСПРЕССИИ мРНК ФАКТОРА НЕКРОЗА ОПУХОЛЕЙ α И ЕГО РЕЦЕПТОРОВ В ЕДИНИЧНЫХ ООЦИТАХ МЫШЕЙ ВО ВРЕМЯ МЕЙОТИЧЕСКОГО СОЗРЕВАНИЯ: РЕЗУЛЬТАТЫ ПОЛИМЕРАЗНОЙ ЦЕПНОЙ РЕАКЦИИ

Исследовано экспрессию мРНК фактора некроза опухолей α (ФНО-α) и его рецепторов I и II типа в единичных ооцитах на стадиях зародышевого пузырька и метафазы II (формирование первого полярного тельца) у мышей линии СВА методом полимеразной цепной реакции. Обнаружена экспрессия мРНК ФНО-α, но не его рецепторов, на обеих стадиях возобновления мейоза, при этом в ооцитах с зародышевым пузырьком ее уровень был достоверно выше, чем в клетках с первым полярным тельцем, что указывает на возможную роль ФНО-α в мейотическом созревании. Полученные результаты являются важным звеном в понимании молекулярных механизмов, регулирующих оогенез и фолликулогенез.

Ключевые слова: фактор некроза опухолей α, рецепторы фактор некроза опухолей α, ооциты, мейотическое созревание ооцитов.

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