

行政院國家科學委員會專題研究計畫成果報告

題目(中文)：性腺激素促進素和卵巢濾泡之成長與萎縮之關係

題目(英文)：GnRH Involvement in Follicular Growth and Apoptosis

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中文摘要

在人類卵巢,引起細胞凋亡的原因並不是很清楚。在本研究裡,我們使用 TUNEL 與 Annexin V/propidium iodide 的染色方法確認人類顆粒黃體細胞有細胞凋亡的現象。新鮮的細胞有 $0.6 \pm 0.2\%$ 凋亡;經過體外培養 48 小時之後則增為 $3.3 \pm 3.0\%$ 。當這些細胞培養過程中同時加入 IFN- γ 與 GnRH agonist 時,凋亡的比率增至 $5.2 \pm 5.1\%$ 。此外,我們發現這些細胞不論新鮮或培養過都會表現 Bcl-2, Fas, FasL, P53 與 CPP32 的 mRNA。但是西方墨點試驗發現只有 Fas, FasL 與 CPP32 在新鮮的細胞有,而培養之後只有 FasL 有出現。我們的結論是細胞凋亡是控制人類顆粒黃體細胞之重要機制之一,而且 GnRH 可以調控這個過程。另外 Fas/FasL, BC1-2, P53 與 CPP32 都是可能調節這些細胞凋亡的基因,其中尤其以 FasL 可能是最重要的因素

英文摘要

The factors that trigger apoptosis are not well

established in human ovary. In this study, we identified the presence of apoptosis in human granulosa-luteal (GL) cells using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and Annexin V/propidium iodide staining methods. It was found that apoptosis was present in fresh GL cells ($0.6 \pm 0.2\%$), and this death phenomenon was increased after in-vitro culture in the absence ($3.3 \pm 3.0\%$; $P=0.03$, compared to fresh samples) or presence ($3.0 \pm 3.9\%$; $P=0.04$, compared to fresh samples) of interferon γ (IFN- γ). This process was further augmented after co-treatment with IFN- γ plus GnRH agonist ($5.2 \pm 5.1\%$; $P=0.04$, compared to IFN- γ alone). In addition, we examined the expression of apoptosis-related genes, including Bcl-2, Fas, Fas ligand, p53, and CPP32 (gene encoding caspase 3) in these cells using Western hybridization and RT-PCR methodology. It was found that transcripts for all the genes that were examined were present in human GL cells. However, only FasL, Fas, and CPP32

proteins were detected in fresh samples, and after culture only FasL remained detectable. It is concluded that apoptosis is one of the potential mechanisms controlling human GL cell development and demise and neuropeptide hormone GnRH analogue is capable of modulating this process. In addition, Fas/FasL, Bcl-2, p53 and CPP32 are among the candidate genes regulating this process, with Fas/FasL likely to be the predominant system.

計劃緣由與目的

Recently, accumulating evidences suggested that follicular atresia and luteal regression occur by a form of programmed cell death, called apoptosis. Though well depicted in animals, the signals responsible for the activation of apoptosis are not yet clear in human ovary. Previous animal studies showed that certain protooncogenes, including Bcl-2 family genes, Fas/Fas Ligand (FasL) system, and p53, are likely to be direct regulators of apoptosis in many cellular systems. Therefore we designed this study to examine the role of these gene expression in the apoptosis of human ovary. It is therefore interesting to examine the expression of these genes, in order to identify their roles in the apoptosis in human GL cells. In addition, a number of reproductive hormones including FSH, LH, GnRH and its agonist (GnRH-a) recently have been introduced into the IVF program for ovulation induction. It was found that the follicular and luteal responses to these chemicals vary greatly among patients. Animal studies also revealed the direct effect

of GnRH and possibly other peptide hormones on the ovary. Since the use of these chemicals significantly affect the outcome of assisted reproductive technology (ART), it is clinically important to identify the effect of these chemicals on the ART. Under the above consideration, this study was designed to examine the status of apoptosis in GL cells and the effect of GnRH-a on this process. Concomitantly, the presence of apoptosis-related genes, including Bcl-2, Fas, FasL, p53, and the downstream effector gene CPP32 was examined in human GL cells. It was hoped that this study can in one aspect help to identify the regulation of apoptosis in human GL cells and in the other to identify the most important apoptosis-related genes in these cells. Additionally, this study may help to establish an in vitro model for future studies on the involvement of various factors (for example reproductive hormones and cytokines) in apoptosis in human follicles and corpus luteum.

結果與討論

Fresh GL cells showed evidences of apoptosis (TUNEL staining positive; TUNEL+)($0.6 \pm 0.2\%$), and this rate increased to $3.3 \pm 3.0\%$ ($P=0.04$, compared to fresh samples) in cells cultured for 48 h in standard media, in cells cultured for 48 h in the presence of IFN- γ ($3.0 \pm 3.9\%$; $P=0.04$, compared to fresh samples), or in cells cultured for 48 h in the presence of GnRH-a alone ($3.2 \pm 2.8\%$; $P=0.04$, compared to fresh samples). This process was further

augmented after co-treatment with IFN- γ plus GnRH-a ($5.2\pm 5.1\%$; $P=0.04$, compared to IFN- γ alone). The data indicate that in vitro culture alone increased the apoptosis rate, and after pre-treatment with IFN- γ , GnRH-a is capable of augmenting apoptosis in human GL cells. RT-PCR technology showed that human GL cells express transcripts of all the genes that were examined, including Bcl-2, Fas, FasL, p53, and CPP32. Semi-quantitation analysis using S26 as an internal control did not show significant difference of these transcript levels among fresh samples and samples after in vitro culture for 48 h in the presence or absence of IFN- γ and/or GnRH-a ($n=5$). However due to the inherent limited sensitivity of this analytical method, a more quantitative method like competitive PCR is needed to confirm this point. Among the proteins selected for screening, Western blotting revealed the presence of Fas (45 Kd), FasL (37 Kd), and CPP32 (caspase 3) (32 Kd) proteins in the fresh samples, with FasL being the predominant species and CPP32 barely detectable. However, only FasL protein was detectable in samples cultured for 48 hours with or without the presence of IFN- γ and/or GnRH-a. P53 was not detectable in fresh and cultured samples. Again no specific change of protein level can be identified before and after culture.

討論

The present study demonstrated that apoptosis was present in human GL cells and certain apoptosis-related genes are expressed. It is thus speculated that apoptosis is likely

one of the mechanism regulating human GL cell development. Among them, Bcl-2, Fas/FasL and p53 are all possible candidate gene, but with Fas/FasL system being the predominant one. This latter system may function through up or down-regulation of the FasL during this period.

In addition, augmented apoptosis after treatment with GnRH-a suggests the involvement of neuroendocrine hormone in the ovarian apoptotic process. The function of GnRH is basically at the level of central nervous system but may also function in the ovary in a paracrine or autocrine manner. Furthermore, potent GnRH analogues have been produced and are frequently used in ART. It is thus interesting to identify the potential effect of GnRH-a on the function and apoptosis of GL cells. The present study therefore confirms the potentially direct effect of GnRH-a in human corpus luteum. These latter approaches will hopefully clarify the roles of reproductive hormones in ovarian apoptosis, and will potentially be capable of improving our control of follicular and luteal function.

Presently, the precise intra-follicular signal(s) that trigger apoptosis of GL cells remain ill defined. However, a number of animal studies suggested that certain proto-oncogenes as Bcl-2 family members and Fas/FasL system may be involved. It is speculated that the balance between these pro- and anti-apoptosis genes expression determines the final fate of a cell. However, the relatively scarce studies in human ovary did not provide a clear enough picture. Representative reports showed that anti-Fas mAb induced apoptosis in human GL cells

and downstream gene products including caspase 1, caspase 3, DNA fragmentation factor, and apoptotic proteinase activating factor-1 were detectable in human GL cells. These latter data raise an interesting but unanswered issue about the comparative significance of the expression of these and related candidate genes in human GL cells. This present study identified the potential involvement of Bcl-2, Fas, FasL, P53, and CPP32 gene expressions in human GL cells. The importance of their roles however needed further evaluation.

Apoptosis is present in a wide variety of cell types. In this study, Bcl-2 protein was not found in fresh or cultured samples, though its transcript was detectable throughout the study period. It is interesting that previous reports about the expression of Bcl-2 transcript and protein in granulosa cells and corpus luteum were also contradictory. For example, Bcl-2 transcript was found in the corpus luteum of rat, but not in cattle. Similarly, Bcl-2 protein was detectable in the rat corpus luteum and chicken ovary, but not in human ovary. Due to the absence of Bcl-2 proteins, this report again can not preclude the possible role of Bcl-2 in GL cell apoptosis, but it raises serious doubt about the significant action of Bcl-2 in the developmental stage of GL cells under study. Since the action of Bcl-2 is predominantly anti-apoptotic, one of the possibilities is that the decreased translation of Bcl-2 may lead to augmented apoptosis in these cells.

In conclusion, the present study demonstrated the presence of apoptosis as one of the possible mechanism regulation GL cell development and regression. This

apoptotic process is augmented after in vitro culture and by the co-treatment with GnRH-a and IFN- γ . Finally, Fas/FasL, Bcl-2, and p53 are all candidate genes in regulating this process, possibly with Fas/FasL playing the major role by up-regulating FasL.

計劃成果自評

Essentially, this study has been conducted according to the original protocol. However, due to the difficulty in setting up the in-vitro perfuse system, part of the original idea must be fulfilled in future work. In total, at least 80% of the expected goal has been achieved and we have obtained interesting and useful information from this study. The observation of the concomitant expression of the spectra of apoptosis-related genes including Bcl-2, Fas/FasL, p53 and CPP32 in human granulosa-luteal cells is novel. These information also greatly enhance our knowledge about ovarian apoptosis. Clinically, this work provides specific guide to our use of GnRH and its analogues in assisted reproductive technology. This latter is especially important since GnRH are so frequently used in ovulation induction in ART. Now the results of this work have been analysed and at a paper (at least) has been written for publication. In conclusion, we feel confident that the project has been satisfactorily finished.

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