表皮生長因子感受器的訊息傳遞在調控成骨細胞分化中所扮演的角色

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中文摘要:

本實驗的目的是要探討表皮生長因子感受器的訊息傳遞在調控成骨細胞分化中所扮演的角色和詳究其功能。近年來的研究顯示出有兩個最主要的訊息傳遞路徑與腫瘤細胞的生長與分化有關聯，它們分別是 JAK/STAT 和 Ras-MAPK 兩大訊息路徑，這兩種訊息路徑可以將細胞外的表皮生長因子訊息藉由其感受器而傳遞進入細胞核內部，進一步來調控該細胞的基因表現；其中 Ras-MAPK 訊息路徑是目前研究最多的訊息路徑，這個路徑調控無數的細胞過程包括細胞增殖和分化。目前有關表皮生長因子如何來調控成骨細胞分化過程的機轉仍不十分明瞭，因此，這個實驗中我們選用了造骨細胞株 MC3T3-E1 (mouse preosteoblastic cell line) 來探討經由表皮生長因子感受器所引發的訊息傳遞路徑。

Abstract:

There is extensive evidence that osteogenic cell differentiation is a multistep series of events mediated by an integrated cascade of gene expression. The aim of this proposal is to investigate the signal transduction pathway mediated by epidermal growth factor receptor (EGF-R) in osteoblastic differentiation. Two major signal transduction pathways involved in cell growth and differentiation have recently been elucidated in tumor cells. These are the JAK (janus kinase)/STAT (signal transducers and activators of transcription) pathway and the Ras-MAP kinase pathway. Among them, the MAP kinase signaling cascade is the best-studied pathway, which regulates a wide array of cellular processes including cell proliferation and differentiation. In this proposed study, we have examined which pathway was involved in EGF-modulated osteoblastic differentiation in preosteoblastic MC3T3-E1 cells.

計劃緣由與目的:

The well-known nature of bone with its active remodeling is indicative of the presence of osteoprogenitor cells, which are destined to osteoblasts and finally osteocytes. Osteoprogenitor cells can be defined as the cell type present in the mature organisms, which has the ability for self-renewal. The bone marrow is known to contain multipotential undifferentiated mesenchymal cells that are able to differentiate

into adipocytes, chondrocytes, fibroblasts, myoblasts and osteoblasts. In order to maintain the phenotype and the population of osteoprogenitor cells, they have to be under negative control so that their differentiation is suppressed. However, this mechanism has not yet been fully characterized. Our previous results demonstrated that all PDL fibroblasts, paravascular cell and preosteoblasts expressed a large number of EGF-R *in vivo*. These observations led us to speculate that even though PDL fibroblasts have the highest synthetic activity of collagen among fibroblasts in oral connective tissues, and have all the morphological characteristics of fully mature fibroblasts, they appear to have some of the properties of undifferentiated cells, at least in regards to EGF-R expression. The EGF-R on these cell types may play a crucial role as a phenotype stabilizer, by functioning as a negative regulator of their differentiation into mineralized tissue forming cells, homeostasis of the fibroblasts population in the PDL, and the maintenance of structural integrity of the periodontium. The role EGF-R in osteogenic cell differentiation was investigated using preosteoblastic MC3T3-E1 cells and osteoblast-like ROS 17/2.8 cells. When cultured in the presence of â-glycerophosphate and ascorbic acid, MC3T3-E1 cells underwent spontaneous differentiation into osteoblasts which was confirmed as they expressed osteoblast markers such as alkaline phosphatase, bone sialoprotein and osteocalcin. Interestingly, the number of EGF-binding sites decreased during their differentiation into osteoblasts, and the osteogenic protein-1 (OP-1) treatment, which accelerated their differentiation, lowered the number of EGF-binding sites even further. On the other hand, ROS 17/2.8 cells with high expression levels of osteoblast markers and no EGF-R, after being transfected with human EGF-R cDNA (designated as EROS cells), expressed numerous EGF-binding sites as well as EGF-R mRNA and protein; in the process, they ceased to express osteoblast markers, indicating their dedifferentiation into osteoprogenitor cells. Both MC3T3-E1 and EROS cells showed increased cell growth in response to EGF, whereas ROS 17/2.8 cells did not. These results imply that EGF-R on osteoprogenitor cell plays a crucial role in the negative regulation of osteoblast differentiation and in the control of self-renewal of the progenitor cell population (Chien *et al.,* 2000).

We have focused our previous work on the investigation of EGF and its receptor in the regulation of periodontal tissue cell differentiation. In this aspect, we have found that EGF-R plays a negative role in regulation of periodontal tissue cell differentiation, especially in osteoblastic cells. The exact mechanisms by which EGF-R functions as a negative regulator in periodontal tissue cell differentiation and maintains structural integrity of the periodontium have not been fully characterized at the present time.

The central theme for this proposed study is to understand the function

of EGF-R and further to explore the EGF-R-mediated signal transduction pathway(s) in osteogenic cell differentiation. The knowledge obtains from this proposed study will help us to better elucidate the roles of EGF and its correspondent receptor in maintenance of bone cell phenotype and in control of self-renewal of osteoprogenitor cells. Information of the EGF-R signaling pathways may help to clarify the mechanisms by which EGF-R acts as a negative regulator of osteoblastic differentiation. By understanding the regulatory mechanisms of osteoblastic differentiation and how progenitor cells remain as an undifferentiated phenotype, we may develop molecular based strategies to recruit and promote mitogenic growth of osteoprogenitor cells, and further increase bone-forming activity via the osteoprogenitor cell cycle.

結果與討論**:**

1. **EGF and GP+AA (b-glycerophosphate and ascorbic acid) up-regulated MAP kinase activity --** MC3T3-E1 cells, a clonal preosteoblastic cell line originated from newborn mouse calvaria (purchased from Riken Gene Bank, Tsukuba Science City, Japan), was plated at 7 x 10^5 cells/60 mm dish and grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (GIBCO/BRL Gland Island, NY, USA). Cultures were grown at 37 °C in a humidified atmosphere of 5% CO_2 until further uses. When grown in the presence of β-glycerophosphate (GP) and ascorbic acid (AA), MC3T3-E1 cells are able to differentiate into osteoblasts and form mineralized bone matrix within 21 days (Chien *et al.,* 2000). Therefore, MC3T3-E1 cells may serve as a useful *in vitro* model of osteoblast development since these cells display a time-dependent and sequential expression of osteoblast characteristics similar to *in vivo* bone formation. To assess the effect of EGF on cell differentiation, MC3T3-E1 cells were cultured on 60-mm culture dishes (Becton Dickson Laboratory) with 10 mM GP and 50 μg/ml of AA in the presence or absence of 200 0r 400 ng/ml EGF (Sigma, USA) for 24 hours. Cell lysates were analyzed for ERK1/2 activation using anti-phospho-specific ERK1/2 antibodies. ECL Western blot demonstrates the effect of EGF on ERK1/2 phosphorylation as indicated in figure 1. However, EGF did not stimulate JAK/STAT activity in MC3T3-E1 cells after 24 hours incubation (data not shown). Thus EGF stimulated ERK of the MAPK superfamily and not the JAK/STAT.

2. **ERK mediated the down-regulation of osteocalcin by EGF –** BMP-2 enhanced the level of osteocalcin expression in MC3T3-E1 cells after 24 hours of incubation.

However, EGF decreased the BMP-2 mediated osteocalcin expression in MC3T3-E1 cells after 3 days of incubation. The effect of EGF on down-regulation of osteocalcin level was attenuated by incubation of the cells with PD98059 (the MEK inhibitor), but not SB203580 (the p38 MAP kinase inhibitor) as shown in figure 2. Our data suggest that EGF down-regulates the osteoblastic cell differentiation probably through the Ras-MAPK, but not the JAK/STAT pathway.

成果自評與未來研究方向:

We have demonstrated that Ras-MAPK signal transduction is induced by EGF in osteoblasts, and the effect of BMP-2 on promoting osteoblastic cell differentiation was attenuated by incubation the cells with EGF. The combined data from our study suggest that the mechanism of EGF on the down-regulation of osteoblastic cell differentiation is via Ras-MAPK pathway.

In our future study, we will investigate whether Ras activity is essential for negatively regulation of osteoblastic cell differentiation. In order to obtain this goal, a constitutively active form of Ras gene (pSV/V_{12} Ras) will be transfected into ROS17/2.8 cells and the osteoblast phenotypic marker will be examined by RT-PCR analysis.

We also will study whether EGF modulates the expression of Cbfa1 in osteoblastic lineage, the expression of mRNA for Cbfa1 will be analyzed by RT-PCR in MC3T3-E1 cells at different stages of osteoblast differentiation, as well as in Ras-transfected ROS17/2.8 cells to determine whether activation of MAP kinase activity alters the expression of Cbfa1 gene. In addition, we will investigate the effects of EGF on OG2 promoter activity in MC3T3-E1 cells. The binding of nuclear extracts obtained from EGF-treated cells to synthetic oligonucleotides containing OSE2 response element will be determined by electrophoretic mobility shift assays. The effects of EGF on osteocalcin gene (OG2) promoter activity will be examined in MC3T3-E1 cells after transfection with luciferase reporter vector containing OG2 promoter region, and the luciferase activity will be measured in these transient transfected cells.

The proposed study will help to better understand the role of EGF-R in maintenance of bone cell phenotype and in control of self-renewal of osteoprogenitor cells. By understanding the regulatory mechanisms of osteoblastic differentiation and how progenitor cells remain as an undifferentiated phenotype, we may develop molecular based strategies to recruit and promote mitogenic growth of osteoprogenitor cells via the osteoprogenitor cell cycle, and further increase bone-forming activity.

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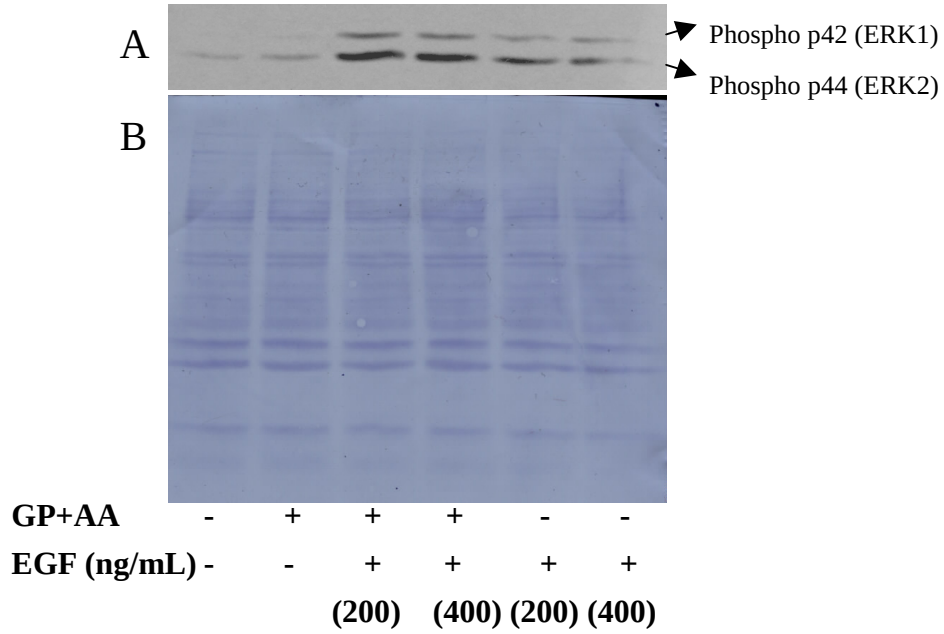

Figure 1: Western blot analysis of ERK1/2 phosphorylation in MC3T3-E1 cells.

A. MC3T3-E1 cells were cultured in defined media indicated above for 24 hours. Cell lysates were analyzed for ERK1/2 activation using anti-phospho-specific ERK1/2 antibodies. ECL Western blot demonstrates the effect of EGF on ERK1/2 phosphorylation.

B. Commassie blue staining of 12% SDS polyacrylamide gel revealed equal amount of proteins from non-treated control and treated cells.

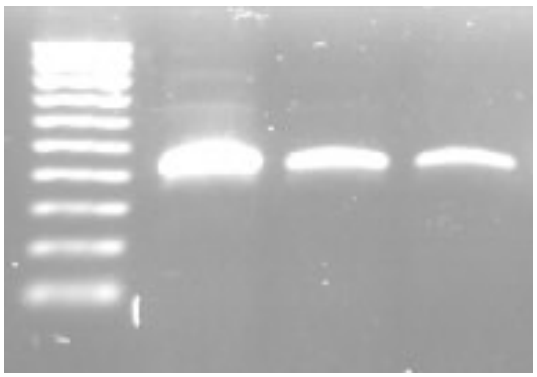

Figure 2: RT-PCR analysis of osteocalcin expression. EGF down-regulated the BMP-2 mediated osteocalcin expression in MC3T3-E1 cells after 3 days of

incubation. (Osteocalcin size: 450 bp)