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EXPERIMENTAL STUDY

Basic fibroblast growth factor upregulates expression of growth hormone gene through extracellular signal-regulated kinase 1/2 in GH4 cells

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Abstract: Basic fibroblast growth factor (bFGF) is reported to not only play multifunctions in pituitary differentiation and tumor formation, stimulating cell differentiation or proliferation, also stimulate pituitary to secret prolactin, growth hormone (GH) and thyroid stimulating hormone, although obvious effect on growth hormone only responded to high-dose bFGF. Since it is well documented that both bFGF and GH correlate closely to tumorigenesis, development and metastasis, so it is necessary to reveal the relationship between the cytokines. In the present report we investigated the effect of bFGF on transcription level of GH gene in GH4 rat pituitary cells as well as the regulatory mechanism with real-time reverse transcription polymerase chain reaction and western blotting. We observed a significant expressional increase of GH gene in GH4 cells stimulated by bFGF, meanwhile phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) was also found in the cells. Further investigation unveiled that PD98059, a specific inhibitor of ERK1/2 signaling pathway markedly impaired the transcriptional increment of GH gene induced by bFGF in the pituitary cells, which indicates that bFGF upregulates GH gene expression through ERK1/2 signaling pathway in GH4 cells. Results may be helpful to elaborate roles of the two cytokines on tumor *(Fig. 3, Ref. 25)*. Full Text in PDF *www.elis.sk*.
Key words: basic fibroblast growth factor, growth hormone, ERK1/2, GH4 cells.

Basic fibroblast growth factor (bFGF), a member of fibroblast growth factor family, has been proved to be a multifunctional biological regulator widely existing in cultured cells, solid tissues, tumors and many organs including pituitary, one of its key functions is stimulating the proliferation of cells from mesodermal and neuroectodermal tissues (1, 2). The cytokine was firstly derived from bovine pituitary, and was found high expression in the follicular cells of pituitary intermediate lobe (2), so some concerned scientists investigated the relationship between bFGF and endocrine system of adenohypophysis. Exposure to bFGF enhances prolactin generation and release from GH3 cells (2), a rat tumoral pituitary cell line, which implies that bFGF receptors are present in GH3 cells, and thus bFGF triggers a series of signaling cascades to perform correlative biofunctions through the surface high affinity receptors of membrane in the pituitary cell. In addition the paper reported a slight increment of transcription of growth hormone (GH) gene in response to high doses of bFGF in the same cells, although the effect was not significant. As a matter of fact, the interaction of the two cytokines not only occurs in pituitary, Izumi et al (3) discovered the upregulation of expression of bFGF gene caused by GH in rat costal cartilage both in vivo and in vitro.

Although an effect of bFGF on expression of GH gene in pituitary cells was reported previously, the deterministic result as well as the possible mechanism through which bFGF works is still veiled. The idea that high-dose bFGF can enhance GH expression lead us to examine facticity of the result, and to further investigate the possible regulatory mechanism. In this study, we administered high-doses of bFGF and examined expression of GH gene in GH4 cells, as well as roles of extracellular signal-regulated kinase 1/2 (ERK1/2) in regulation of bFGF on expression of GH gene.

Materials and methods

Cell culture

GH4 cells, a rat tumoral pituitary cell line which produces GH, were grown in Dulbecco's modified Eagle's medium (DMEM) and Han F-12 (DMEM/F12=1:1) supplemented with 10 % fetal bovine serum (FBS) until confluence at 37 °C with 5 % CO_2 in a humidified atmosphere. Cells were harvested by trypsinization and seeded in dishes (5 cm in diameter) at a density of $1x10^5$ cells in 1 ml of culture medium, when cells were grown to 75–80 % confluence, medium was replaced with DMEM/F12 containing 0.5 % FBS, and cells were incubated for 48 h before experiment.

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Quantitative RT-PCR

Total RNA was extracted and used to assess the relative abundance of mRNAs between GH gene and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) gene in GH4 cells by real-time reverse transcription polymerase chain reaction (RT-PCR). The quantitative RT-PCR was performed using SYBR GREEN PCR master mix (ABI Applied Biosystems, Foster City, USA) on an ABI PRISM 7900 Sequence Detector System. Annealing temperatures and primer sequences of GH and GAPDH genes were as follows. GH gene: primers 5'-CTA TCA GAG AGT AGA GAC GCC AGT-3' and 5'-GGG AAT GGA ATG GCT GTA GTC-3', annealing temperature 61 °C; GAPDH gene: primers 5'-TGA TGA CAT CAA GAA GGT GGT GAA G-3' and 5'-TCC TTG GAG GCC ATG TAG GCC AT-3', annealing terperature 62 °C. GH mRNA level was normalized with GAPDH mRNA level.

Western blotting analysis

Cells were lysed in ice-cold non-denaturing cell lysis buffer, the harvested lysate was denatured in protein sample buffer for 5min in boiling water. After sodium dodecylsulfate polyacrylamide gel electrophoresis, protein was transferred to nitrocellulose membrane and then probed with monoclonal antibody, against extracellular signal-regulated kinase 1/2 (ERK1/2, CST, Boston, USA) or phosphate extracellular signal-regulated kinase 1/2 (p-ERK1/2, CST, Boston, USA), that would be captured by the secondary antibody conjugated with horseradish peroxidase(Santa Cruz, USA). The membrance was visualized with SuperSignal West Pico chemiluminesence detection system (Pierce, Rockford, USA).

Statistical analysis

Value is expressed as mean ± standard error (SEM). The significance between treatment and control cells was determined by paired Student's *t*-test. $P<0.05$ was set to indicate the significant difference statistically.

Results

Basic FGF increased expression of GH gene in GH4 cells

It was reported unobvious effect of low-dose of bFGF on GH gene expression (2), to test whether high-dose of bFGF stimulates significant gene expression, this paper scheduled three doses of bFGF to treat GH cells in vitro. Results present in Figure1 shows that low-dose of bFGF (30 ng/ml) could not change expressional level of GH gene in GH4 cells, while compared with untreated cells, GH mRNA expression reached a 4.44-fold increase even 2 h after treatment with high-dose of bFGF (60 ng/ml), and declined to 1.75-fold increment as bFGF concentration increased to 100 ng/ml. This pattern implies a dual modulation of exogenous bFGF on transcription of endogeneous GH gene.

PD98059 inhibited bFGF induced activation of ERK1/2 in GH4 cells

Presently no relevant information about regulatory mechanism of bFGF on GH gene expression. Basic FGF receptor tyrosine kinase links to the G-protein Ras, which stimulates the MAPK

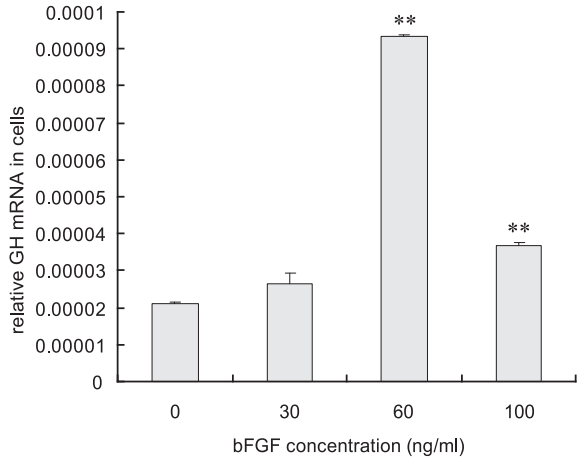

Fig. 1. Effects of different concentrations of bFGF on expression of GH gene in GH4 cells. The cultured GH4 cells were grown in DMEM/F12 containing 0.5 % FBS for 48 h and then transferred into DMEM/F12 containing 0.5 % FBS, 10 ng/ml heparin and bFGF at the indicated concentrations for 2 h. Total mRNA was extracted from cells and subjected to determine GH gene transcriptional level with real-time RT-PCR. Values were expressed as the means ± standard errors. ** p<0.01, vs vehicle treated cells.

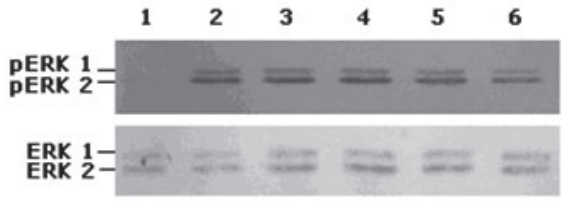

Fig. 2. Activation of ERK1/2 induced by bFGF in GH4 cells. GH4 cells were grown in DMEM/F12 containing 0.5 % FBS for 48 h and transferred into DMEM/F12 containing 0.5 % FBS, 10 ng/ml heparin and 60 ng/ml bFGF for indicated time. Cell lysate was subjected to western blot analysis. Lane 1 to 6 represent different treatment time, 0 min, 5 min, 10 min, 20 min, 30 min and 60 min, respectively.

signal transduction cascade. Among the MAPKs, ERK1/2 play a pivotal role in mediation of cellular responses to signaling molecules, so we want to know whether the signaling pathway MEK/ERK-MAPK is also selected by bFGF to perform its biological functions in GH4 cells. Results described in Figure 2 reveals that, at the time of initial treatment the phosphorylated level of ERK1/2 was far below that occurred in other indicated time, which showed that bFGF (60 ng/ml) obviously caused the phosphorylation of ERK1/2 during the scheduled stimulating time, from 5 min to 60 min. Figure 2 shows us an initial rapid phosphorylation and a gradual increase activity induced by bFGF, and the maximum activity at approximately 20 min.

PD98059 impaired improvement of bFGF on transcription of GH gene in GH4 cells

To investigate whether ERK1/2 activated by bFGF participates in the regulatory mechanism of the growth factor improving expression of growth hormone gene in GH4 cells, PD98059 was used to pretreat the rat pituitary cells before treatment with

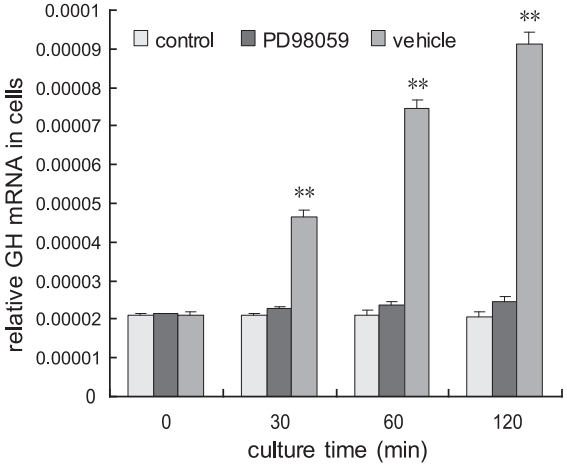

Fig. 3. Inhibition of PD98059 on transcriptional increment of GH gene induced by bFGF in GH4 cells. GH4 cells were grown in DMEM/F12 containing 0.5 % FBS and 50 μM PD98059 or vehicle for 60 min and transferred into DMEM/F12 with 0.5 % FBS, 10 ng/ml heparin and 60 ng/ml bFGF and incubated for indicated time. Control cells were cultured in DMEM/F12 with 0.5 % FBS. Values were shown as the means ± standard errors. ** p<0.01, vs PD98059 treated cells.

bFGF. Data present in Figure 3 show that the inhibitor markedly suppressed the transcriptional increment of endogeneous GH gene induced by additional bFGF, which indicated an indubitable role of ERK-MAP-Kinase in regulatory process of bFGF enhancing expression of GH gene in GH4 cells.

Discussion

Basic FGF, one of 22 members of FGF families, was firstly isolated as mitogens from bovine pituitary in the 1970s, but Baird et al found a nonmitogenic function of improving expression of thyrotropin and prolactin genes in the brain tissue (4), which unclosed a relationship between bFGF and pituitary endocrine system. Up to now, little information about the connection of bFGF and GH has been shown to us, the objectivity has been already approved by few studies (2, 3). Although Izumi et al reported a marked improvement of GH on expression of bFGF gene in rat costal cartilage both in vivo and in vitro, no further investigation occurred in the interaction between bFGF and GH in pituitary except for a unobvious expressional enhancement of GH gene induced by high-dose of bFGF (40 ng/ml) in GH3 cells. In this paper, we employed higher doses of bFGF (60 ng/ml and 100 ng/ml) to stimulate GH4 cells in vitro, results revealed that the two scheduled higher doses of bFGF, especially 60 ng/ml, significantly upregulated endogeneous transcription of GH gene in the rat pituitary cell line.

As a mitogen and differentiation factor for neuroectoderm- or mesoderm-derived cells, bFGF has been demonstrated to be a multifunctional cytokine, especially its potent tumorigenic activity (5, 6). Large numbers of background researches revealed that bFGF stimulated cells proliferation and angiogenesis in tumor and involved in tumor growth, metastasis and prognosis through autocrine and paracrine manners, thus the cytokine has been regarded as a target molecule for curing cancers by concerned scientists. Some bioremediation approaches aimed at bFGF have been confirmed to be effective to suppress the proliferation of tumor cells, such as antibodies against bFGF (7, 8), antisense bFGF gene (9), RNase-1 conjugated to bFGF (10), inhibitor of bFGF-induced signal pathway (11–15), mimic peptide to bFGF (16) and other cytokines (17, 18). In addition, as an important regulator in process of postnatal growth, development and metabolism, GH has been found high expression in various tumors, and the expressional level varies with tumor pathological stage and degree of differentiation (19), which implys that GH maybe correlate to tumorigenesis, development and metastasis. From above information about the two cytokines and our findings in this paper, we easily suppose that, during the process of bFGF's positive regulation on tumor, it is on the cards that GH is a downstream effector of bFGF. So we can reasonably deduce a possible pathway of bFGF's roles on tumor through stimulating GH that in turn plays bioactive function by activating IGF-1 (insulin-like growth factor 1, a critical unamiable cytokine in tumor) pathway and IGF-1-independent pathway (20–22).

It is known that the pleiotropic effects of bFGF are transduced by its interaction with any one of four members of a family of high-affinity cell surface receptors (FGFR1 to FGFR4), bFGF induces FGFR dimerization and subsequent transphosphorylation which triggers a series of signaling transduction cascades. Among all of the FGFRs and their different splice variants, bFGF usually activates FGFR2-IIIc (23). Jackson et al (24) found that GH4 rat pituitary cells expressed all four FGFRs, and bFGF phosphorylated the tyrosines of FGFR1, FGFR3 and FGFR4, but not FGFR2, and resulted in no significant change in GH4 cell number, which implys that bioactivity of bFGF on GH4 cell is not mitogenic but regulating neuroendocrine. Background researches have proved that bFGF stimulates PRL, GH and TSH secretion, the effect on PRL being the most prominent (25). Basic FGF triggered signaling transduction cascades includes MAPK and so on. Basic FGF receptor tyrosine kinase links to the G-protein Ras, which stimulates the MAPK signal transduction cascade. Among the MAPKs, ERK1/2 plays a pivotal role in mediation of cellular responses to signaling molecules. Ras activates MAPK and in turn activates ERK1/2, the activated ERK1/2 plays its biological roles. In GH4 rat pituitary cells bFGF markedly activated MAPK and induced prolonged the kinases activation (24), the results reappeared in our present experiment, we revealed the phosphorylation of ERK1/2, a member of MAP-Kinases, by bFGF in GH4 cells, and transcriptional increment of GH gene responses to bFGF with respect to activation of the ERK1/2. We propose that ERK signaling pathway must be a vital mode of bFGF-induced increasing expression of GH gene, which may be an important cause of bFGF's positive action on tumor generation and progression.

Summarily, in the present paper, we showed that bFGF upregulated expression of GH gene through ERK1/2 signaling pathway in GH4 cells, and deduced that expressional increment of the hormone gene may be an important reason in the process of bFGF stimulating tumorigenesis, development and metastasis. However

this deduction is only founded on the interaction of the two cytokines in GH4 cells, in fact both GH and bFGF have been found high expression in various tumors, and the expressional level varies with tumor pathological stage and degree of differentiation, so we think investigation on effects of bFGF on expression of GH gene in various tumor cells must be more important and more effective in elaborating our deduction.

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