Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Effects of recombinant human growth hormone on proliferation and differentiation of cementoblast and ERK1 / 2, JNK / SAPK and p38MAPK signaling pathway

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ARTICLE INFO

Original paper

Article history:
Received: March 12, 2022
Accepted: June 17, 2022
Published: July 31, 2022

Keywords:

Recombinant human growth hormone, proliferation and differentiation of cementoblast, ERK1 / 2, JNK / SAPK, p38MAPK

ABSTRACT

To investigate the effects of recombinant human growth hormone on the proliferation and differentiation of cementoblast and the signal pathways of ERK1 / 2, JNK / SAPK and p38MAPK, osteoblasts (OCCM-30) were cultured in vitro. The OCCM-30 was treated with different concentrations of recombinant human growth hormone (rhGH) (0, 10, 50 ng/mL) for 1 d and 2 d, respectively. MTT assay was used to test the proliferation of OCCM-30 cells by rhGH. The effect of BSP, OPN, OCN and ALP genes was detected by RT-PCR after one day. The activity of alkaline phosphatase (ALP) was detected after treatment with OCCM-30 for five days at different concentrations of rhGH. After treatment with OCCM-30 at 100 ng/mL rhGH for 0 min, 5 min, 10 min, 15 min, 30 min and 60 min, the phosphorylation levels of ERK1/2, JNK/SAPK and p38MAPK were detected by Western blot. Results showed that rhGH could promote the proliferation of OCCM-30 cells, and the proliferation of OCCM-30 cells increases with the increase of rhGH concentration. After one day of culture, the levels of the BSP and ALP genes increased with the increase of rhGH concentration ($P<0.05$); the OPN gene level in the 10 ng/mL group was significantly higher than that in the blank group, and the 50 ng/mL group was significantly lower than the blank group. ($P<0.05$); OCN gene levels in the 10 ng/mL group and 50 ng/mL group were not significantly different from those in the blank group ($P>0.05$). Compared with the blank group, the ERK 1/2 phosphorylation level increased at 5 min in the 100 ng/mL group, reached the maximum at 10 min, decreased significantly at 15 min, decreased to the original level at 30 min, and had no significant change in ERK 1/2 total protein level; 100 ng/mL rhGH had no significant effect on SAPK/JNK, p38MAPK phosphorylation and total protein levels in OCCM-30 cells ($P>0.05$). It was concluded that 10ng/mL and 50ng/mL rhGH could promote the proliferation of OCCM-30 cells and promote the expression of the BSP gene and ALP gene. Low-dose rhGH is beneficial to OPN gene expression, and high-dose rhGH inhibits OPN gene expression. 100 ng/mL rhGH promoted the ERK 1/2 pathway and had no effect on the SAPK/JNK and p38 MAPK pathways.

Doi: http://dx.doi.org/10.14715/cmb/2022.68.7.8

Introduction

The cementum is a layer of mineralized tissue that covers the surface of the root and consists of cells and intercellular substances. As people's living standards improve, oral health awareness increases, and people pay more and more attention to cementum. Cementoblasts (CB) are important functional cells in the cementum. CB expresses bone sialoprotein (BSP), osteopontin (OPN), osteocalcin (OCN), etc. Mineralization-related proteins play an important role in the process of root formation, absorption and repair (1). ERK1/2, JNK/SAPK, and p38MAPK are members of the MAPKs family and are a common pathway in a variety of signal transduction processes, playing a key role in cell proliferation, differentiation, and apoptosis (2). By activating/inhibiting the MAPKs signaling pathway, multiple extracellular stimuli can affect the biochemical activity inside the cell. In recent years, researchers have found that rhGH promotes cementation and stimulates MAPKs signaling pathway (3-4). At present, rhGH has little effect on the proliferation, differentiation and ERK1/2, JNK/SAPK and p38MAPK signaling pathways of CB. This study focused on the effect of rhGH on CB proliferation, differentiation and ERK1/2, JNK/SAPK, p38MAPK signaling pathway and provided an experimental basis for the clinical repair of cementum.

Materials and Methods

Main experimental materials and instruments

Osteoblasts (OCCM-30), DMEM/F12 medium (Shanghai Biochemical Reagents Co., Ltd.), recombinant human growth hormone (rhGH) (Shanghai United Saier Bioengineering Co., Ltd.), bovine serum albumin (BSA)), fetal bovine serum (FBS), penicillin/streptomycin (P/S), trypsin (Shanghai Kenqiang Instrument Co., Ltd.), dimethyl sulfoxide (DMSO, Shanghai Kenqiang Instrument Co., Ltd.), methanol, ten Sodium dialkyl sulfonate, glycine, paraformaldehyde, absolute ethanol, chloroform, isopropanol, ascorbic acid, β-glycerophosphate disodium salt

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hydrate, alizarin red, 5× loading buffer (Wuhan Google biotechnology) Co., Ltd., protein lysate (including protease inhibitor, phosphatase inhibitor) (Biyuntian Biotechnology Research Institute), rabbit ERKI/2 polyclonal antibody, rabbit p-ERKl/2 monoclonal antibody, rabbit P38 polyclonal antibody , rabbit p-p38 polyclonal antibody, rabbit SAPK/JNK polyclonal antibody, rabbit p-SAPK/JNK polyclonal antibody, rabbit GAPDH polyclonal antibody, horseradish peroxidase-labeled goat anti-rabbit IgG (American cell signaling technology co.), alkaline phosphatase activity measurement Box, Reverse Transcription Kit (Qiyi Biotechnology (Shanghai) Co., Ltd.), MTT Cell Proliferation and Cytotoxicity Assay Kit (Biyuntian Biotechnology Research Institute), Protein Quantification Kit (BCA) (Beijing Pulilai Gene) Technology Co., Ltd.), PCR instrument (Chengdu Besida Instrument Co., Ltd.), SYBR quantitative PCR kit (TaKaRa), primer (Bioengineering (Shanghai) Co., Ltd.), SYBR fluorescence quantitative PCR instrument (ABI, USA) Microplate reader (Jinan Baihe Medical Instrument Co., Ltd.), CO2 cell incubator (Shanghai Qiaoyue Electronic Technology Co., Ltd.), Western Blot Luminal Reagent (United States Thereto scientifi company), developer, fixer (Wuhan Google Biotechnology Co., Ltd.)), glue machine, electrophoresis tank, electrophoresis instrument (BIO-RAD, USA).

Experimental group: blank group (normal medium), experimental group (normal medium + rhGH), 3 replicates in each group. The rhGH concentrations in the experimental group were 10 ng/mL and 50 ng/mL, respectively.

Primary culture of OCCM-30

After OCCM-30 was rewarmed, it was sucked into a centrifuge tube, mixed with DMEM, and the supernatant was discarded. Add DMEM medium containing 10% FBS, mix well, inoculate in 25cm2 cell culture flask, and incubate in CO2 incubator (37 °C, 5% CO2, 95% air), change every 2d (including 10% FBS)). When the cells are covered with 70%~80% of the bottom of the bottle, they can be passaged, and 3~5 generation OCCM-30 cells are used for the experiment.

MTT assay for OCCM-30 cell proliferation

OCCM-30 was inoculated into 96-well plates at 3×10^3 cells/well, and each group had 3 replicate wells. The cells were adhered to the cells and cultured in a DMEM medium without FBS for 24 hours. Then different concentrations of rhGH (0, 10, 50 ng / mL) DMEM medium were exchanged. The absorbance value (OD) was measured after 1 d and 2 d of culture. Before the measurement, 20 μL of 5 mg/mL MTT was added to each well, and after incubating for 4 hours in the incubator, the supernatant was discarded, 150 μL of DMSO was added to each well, and the shaker was shaken for 20 min. After the crystal was fully dissolved, the wavelength of 570 nm was measured by a microplate reader for OD value.

Alkaline phosphatase (ALP) activity assay

OCCM-30 was inoculated into 96-well plates at 3×10^5 cells/well, and each group had 3 replicate wells. The cells were adhered to the cells and cultured in a DMEM medium without FBS for 12 hours. Then, different concentrations of rhGH (0, 10, 50 ng / mL) DMEM medium were exchanged, and the culture medium was changed every 2 days. After 5 days of culture, the supernatant was discarded, washed three times with PBS, and 50 μL of 1% Triton-X100 was added to each well. After incubating for 2 h in the incubator, ALP substrate was added (operating according to the ALP kit instructions), and the OD560 value at a wavelength of 570 nm was measured with a microplate reader. At the same time, the cell protein concentration was measured by the BCA method.

RT-PCR detection

OCCM-30 was inoculated into 6-well plates at 3×10^5 cells/well, and each group had 3 replicate wells. The cells were adherent and cultured in a DMEM medium without FBS for 24 hours. Then different concentrations of rhGH (0, 10, 50 ng / mL) DMEM medium were exchanged. After 1 day of culture, total RNA was extracted and then reverse transcribed to obtain cDNA, and the primers were synthesized by Bioengineering (Shanghai) Co., Ltd. (Table 1), followed by real-time PCR. The amplification conditions were as follows: denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, extension at 72 °C for 30 s, and amplification for 40 cycles. The analytical data were collected and the relative copy number of the gene to be tested was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot detection

OCCM-30 was inoculated into 6-well plates at 3×10^5 cells/well, and each group had 3 replicate wells. The cells were adhered to the cells and cultured in a DMEM medium without FBS for 12 hours. Then, different concentrations of rhGH (0, 100 ng / mL) DMEM medium (such as adding MAPKs pathway inhibitor, then add rhGH after 1h). After 2 days of culture, the supernatant was discarded, washed three times with PBS, and 200 μL of protein lysate was added to each well. After placing on ice for 5 min, the mixture was collected by EP tube, centrifuged at 4 °C 12000 r/min for 10 min after ultrasonic sonication, and the supernatant was taken. The protein concentration was measured by the BCA method, and the protein concentration of the sample to be tested was adjusted. Add protein loading buffer, mix and boil for 5 min, and store at -40 °C. The sample protein was separated by electrophoresis according to the instructions of the SDS PAGE gel kit, 5%

Table 1. RT-PCR upstream and downstream primers.

Gene	Primer sequence	
	Transcription (5'-3')	Reverse transcription (5'-3')
GAPDH	CCACTCTTCCACCTTCG	GTGGTCCAGGGTTTCTTAC
BSP	TCAGAAAAAGCAGCACCG	TCGTAGCCTTCATAGCCAT
OPN	TTTCACTCCAATCGTCCCTA	CCTTTCCGTTGTTGTCCTGA
OCN	TAAGGTAGTGAACAGACTCCG	CCGTAGATGCGTTTGTAGG
ALP	TCCCTACCGACCCTGTTCTGA	TGGACCTCTCCCTTGAGTGT

separation gel and 10% separation gel, 10 μL/lane loading. 210 mA constant current transfer film for 90 min, remove PVDF membrane, 5% skim milk powder closed for 1 h, TBST shake for 5 min × 3, primary antibody 4 °C shaker overnight, next day second anti-room temperature 1 h, TBST shake 5 min × 3, add chemiluminescence The substrate was incubated for 3 min and a chemiluminescent gel imaging system was used for exposure imaging.

Statistical analysis

The data in this paper were collected by the participating researchers. After the statistics, the data was completely entered into the research database. The data of this survey was statistically analyzed using SPSS21.0 software. The count data were expressed as a percentage. The comparison was performed using the χ^2 test, and the measurement data was indicated by "$\bar{x} \pm s$". The t-test was used for comparison. The test level was P<0.05, indicating that the data comparison results were statistically significant.

Results

The effect of rhGH on the proliferation of OCCM-30

The results of the MTT test showed that the OD value of OCCM-30 in the experimental group was significantly higher than that in the blank group after 1d and 2d, and the difference was statistically significant (P<0.05). The 10ng/mL group and the 50ng/mL group in the experimental group. The difference between the two groups was statistically significant (P<0.05), as shown in Table 2.

Effect of rhGH on OCCM-30 ALP activity

Compared with the blank group, the ALP activity of the cells increased significantly after 5 days of 10ng/mLrhGH treatment. The ALP activity of the cells decreased significantly after 5d of 50ng/mL rhGH for 5 days. The difference was statistically significant (P<0.05), and the 10ng/mL group and 50ng after 5d. The ALP of the /mL group was statistically significant (P<0.05).

The effect of rhGH on the differentiation of OCCM-30

After 1 day of culture, the BSP gene increased with the increase of rhGH concentration. The difference between the experimental group and the blank group was significant (P<0.05). There was no significant difference in the expression of the BSP gene between the 10ng/mL group and the 50ng/mL group (P>0.05). The expression level of the OPN gene was significantly higher in the 10 ng/mL group than in the blank group and 50 ng/mL group, and the 50 ng/mL group was significantly lower than the blank group (P<0.05). The OCN gene expression level was compared. There was no significant difference between the 10 ng/mL group and the 50 ng/mL group and the blank group (P>0.05). The 10 ng/mL group was significantly different from the 50 ng/mL group (P<0.05). The ALP gene was associated with rhGH concentration. The increase was significantly higher in the experimental group than in the blank group (P<0.05). There was no significant difference between the 10 ng/mL group and the 50 ng/mL group (P>0.05), as shown in Table. 3.

Effect of rhGH on the MAPKs signaling pathway

Compared with the blank group, the ERK 1/2 phosphorylation level increased at 5 min in the 100 ng/mL group, reached the maximum at 10 min, decreased significantly at 15 min, decreased to the original level at 30 min, and had no significant change in ERK 1/2 total protein levels; 5 min, 10 min At 15 min and 30 min, there was no significant change in SAPK/JNK, p38 MAPK phosphorylation and total protein levels in the 100 ng/mL group, as shown in Table 4.

Discussion

CB is the basis of cement restoration and regeneration. It can secrete a cementum matrix and form repaired cementum on the surface of the root. CB can also inhibit root resorption by inhibiting the adhesion of osteoclasts (5,6). The CB used in this study was OCCM-30 cells derived from mouse osteoblastic cell lines, which could proliferate without altering hereditary traits, and could exclude the interference of primary cell defects on the experiment.

rhGH is a protein consisting of 191 amino acid residues that regulate the endocrine system, activates and maintains the normal function of the immune system. In 1958, natural human growth hormone (ph-GH) was used to treat children with pituitary dwarfism. In 1985, it was found that after using recombinant growth hormone, the anti-

Table 2. Effects of different concentrations of rhGH on the proliferation of cementoblasts.

Group	0ng/mL	10ng/mL	50ng/mL
24h	0.12±0.03	0.18±0.04	0.25±0.05
48h	0.35±0.10	0.56±0.11	0.67±0.14

Table 3. Effects of different concentrations of rhGH on the expression of osteoblast-associated genes in dental cement

related gene	0ng/mL	10ng/mL	50ng/mL
BSP	1.10±0.09	4.38±0.34	5.37±0.82
OPN	1.08±0.10	1.67±0.15	0.44±0.06
OCN	1.06±0.03	0.83±0.05	1.36±0.08
ALP	1.07±0.05	1.85±0.17	1.32±0.26

Table 4. Effect of 100ng/mL rhGH on MAPKs signaling pathway in cementoblasts.

Related proteins	0min	5min	10min	15min	30min	60min
p-ERK1/2	0.08±0.01	2.86±0.30	5.64±0.56	0.24±0.05	0.06±0.03	0.05±0.02
ERK1/2	1.07±0.09	1.08±0.03	1.05±0.05	1.03±0.06	1.05±0.06	1.04±0.03
p-SAPK/JNK	0.34±0.03	0.30±0.04	0.35±0.06	0.37±0.06	0.36±0.04	0.31±0.05
SAPK/JNK	1.07±0.05	1.06±0.12	1.06±0.09	1.03±0.07	1.05±0.08	1.07±0.06
p-p38MAPK	0.25±0.03	0.21±0.04	0.27±0.05	0.26±0.06	0.23±0.07	0.28±0.06
p38MAPK	1.02±0.10	1.05±0.09	1.04±0.08	1.02±0.12	1.05±0.07	1.08±0.08

hGH antibody productivity in humans can reach 64%. In the 1980s, rhGH was marketed, and its biological activity was the same as that of natural h-GH, and the antibody production rate was less than 1%. At present, rhGH is used clinically to treat adult growth hormone deficiency, acute necrotizing pancreatitis, cardiovascular disease and postoperative respiratory failure. At the same time, a large number of studies have found that rhGH can promote bone cement formation (7). Smid et al (8) reported that the length and width of cellular cementum were affected by GH levels. In this study, the effect of different concentrations of rhGH on the proliferation of OCCM-30 cells was detected by MTT assay. The results showed that the proliferation of OCCM-30 was affected by rhGH concentration. With the increase of rhGH concentration, the proliferation rate was faster, suggesting that rhGH promoted the proliferation of CB. The effect is similar to that of Hu Yajun et al. (5).

ALP plays an important role in biomineralization and bone calcification. It is a marker protein that differentiates cells into osteogenesis. As the degree of cell differentiation deepens, ALP expression is enhanced, and ALP activity can mark the differentiation activity of bone-forming cells(9). Dalla-Bona et al (10) believe that ALP can be used as an early marker of CB. This study showed that ALP activity in OCCM-30 cells was affected by rhGH concentration, 10 ng/mL rhGH enhanced cell ALP activity, and 50 ng/mL rhGH decreased cell ALP activity.

BSP is related to the mineralization of CB. Some studies have found that the expression level of BSP is increased when the cementum begins to mineralize (11). OPN plays a role in regulating the growth rate of nucleation growth in CB mineralization. OCN is an important marker of bone formation and can be used as an indicator to reflect the differentiation and maturation of CB (12). Studies have found that a variety of factors can affect the expression of these genes in cementoblasts, such as dexamethasone, tyrosine-rich enamel protein, β-transforming growth factor, and hypoxia (13). In this study, RT-PCR was used to detect the effect of rhGH on the expression of BSP, OPN, OCN and ALP. The results showed that the expression level of the BSP gene increased with the increase of rhGH concentration; 10ng/mL rhGH could enhance the expression level of the OPN gene, 50ng/mL rhGH attenuated OPN gene expression; 10ng/mL and 50ng/mL rhGH had no effect on OCN gene expression; The ALP gene increases with increasing rhGH concentration.

The MAPKs signaling pathway is a relay station for multiple signaling pathways within cells. ERK1/2, SAPK/JNK and p38MAPK are important members of the MAPKs family, and these three pathways interact and regulate each other. ERK1/2 has a regulatory effect on biological activities such as cell proliferation, differentiation and apoptosis (14). JNK mainly exists outside the nucleus and is transferred to the nucleus after external stimulation, which changes the expression of related genes. The SAPK/JNK signaling pathway plays a regulatory role in inflammation, neurodevelopment and apoptosis (15). P38MAPK phosphorylates under certain external stimuli, transfers to the nucleus, stimulates the expression of multiple transcription factors in the nucleus, and participates in cell biochemical reactions. The auxin mainly regulates the growth and metabolism of the body through the JAK2-STATs signaling pathway. It has been found that MAPKs are also involved in this process (16). This study showed that 100 ng/mL rhGH promoted ERK 1/2 phosphorylation and had no effect on SAPK/JNK, p38MAPK phosphorylation and total protein levels.

In summary, 10 ng/mL, and 50 ng/mL rhGH can promote the proliferation of OCCM-30 cells and promote the expression of the BSP gene and ALP gene. Low-dose rhGH is beneficial to OPN gene expression, and high-dose rhGH inhibits OPN gene expression. 100 ng/mL rhGH promoted the ERK 1/2 pathway and had no effect on the SAPK/JNK and p38 MAPK pathways.

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