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Mechanism of BMP and TG2 in mesenchymal stem cell osteogenesis

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Abstract. – OBJECTIVE: To study the interactive effects of Type II glutamine transaminase (TG2) and bone morphogenetic protein-9 (BMP-9) in the induction of osteogenesis in mice mesenchymal stem cells (MSCs) C3H10T1/2 model.

MATERIALS AND METHODS: Batches of MSCs C3H10T1/2, divided into two groups, were treated with BMP-9 (control group) or BMP-9 and TG2 (experimental group) under oxygen deficient conditions. The secreted alkaline phosphatase (SEAP) chemiluminescence and the histochemical staining methods were used to detect the alkaline phosphatase (ALP) expression. The alizarin red S staining was used to detect the calcium salt precipitation and the caspase-3 protein expression was monitored using Western blot. Flow cytometry was employed to identify cell cycle, and trypan blue exclusion method to count the living cells and monitor cell proliferation.

RESULTS: The levels of ALP expression in the experimental group were much higher than that of the control group. The level of expression of advanced caspase-3 protein was significantly lower ($p < 0.05$) in the experimental group than in the control group. The highest fraction of cells in the experimental group was in the phase M while cells in the control group were in the interphase. Moreover, cell number in the experimental group was significantly increased ($p < 0.05$) relatively to the control group.

CONCLUSIONS: BMP-9 interacts with TG2 in osteogenesis of MSCs C3H10T1/2 cells. Further studies are needed to understand the exact mechanism of BMP9/PG2 interactions in osteogenesis.

Key Words:
BMPs, TG2, Mesenchymal stem cell, Osteogenesis.

Introduction

Bone undergoes continuous remodeling throughout life[1] through an unknown complex and well orchestrated process of osteoblast lineage specific differentiation[2]. Indeed, during osteogenesis, pluripotent stem cells undergo successive stages of differentiation with decreasing potential of proliferation leading to subsequent pre-osteoblast differentiations into mature osteoblast that deposit to form bone matrix[2], following by mineralization[2].

Though the elucidation of detailed molecular events underlying the osteogenic processes still ongoing, bone morphogenetic proteins (BMPs) members which belong to the transforming growth factor β (TGF-β) superfamily[3-6] have been shown to play important role in the regulation of osteoblast differentiation and subsequent bone formation[7,8]. Indeed, BMPs are involved in bone and cartilage formation[6,8], and are multifunctional regulators of proliferation and differentiation during development[9,10]. Accordingly, BMP-9, also termed growth differentiating factor 2, exhibits strongest osteogenic induction ability[11] compared with BMP-2 and BMP-7[12,13]; particularly in mesenchymal stem cells (MSCs), the bone marrow progenitor cells with the capacity to differentiate along osteogenic, chondrogenic, myogenic and adipogenic lineages[11].

In line with these data, adenoviral expression of BMP-9 in MSCs increases the expression of osteogenic markers and induces tubular bone and osteoid matrix formation[11]. BMP-9 exhibited significant molecular cross-talk with several signaling pathways thereby by acting synergically in bone formation with some of these signaling pathways, including Wnt/-β-catenin, IGF, retinoid signaling pathways[14], and possibly transglutaminase 2 (TG2), also known as tissue transglutaminase. TG2 is involved in the mediation of hypertrophic differentiation of joint chondrocytes and interleukin-1-induced calcification[15]. Consistently, TG2 has been found to mediate cross-linking of BMP-2 to induce osteoblast-specific programming of human bone marrow-derived mesenchymal stem cells (hBMSCs)[16]. But there is no data show-

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ing whether TG2 also interact with BMP-9 during osteogenesis. We then addressed this question by treated MSCs C3H10T1/2 with BMP-9 alone (control group) or in combination with TG2 (experimental group) under oxygen deficient conditions. TG2 could interact and combine with caspase-3 to consume caspase-3 as observed in anoxic tumor cells and thus preventing cell apoptosis[17]. MSCs C3H10T1/2 used coming from mice embryo stem cell line separated by Reznikoff et al from C3H mouse. Its multiple differentiations potential is comparable to BMSCs and its adipogenic differentiation is relatively low. The cells are stable and homogenized and have no spontaneous differentiation under general culturing conditions[18]. In bone tissue engineering, C3H10T1/2 is a favorable substitute for BMSCs.

Materials and Methods

Animals

The murine mesenchymal stem cell C3H10T1/2was purchased from the Bone and Molecular Cancer Research Center of the University of Chicago, IL, USA. The Luciferase reporter gene plasmid p12xSBE-Luc was constructed and preserved by the Bone and Molecular Cancer Research Center of the University of Chicago. The adenovirus vector AdBMP9 (with GFP expression) was built by the Bone and Molecular Cancer Research Center of the University of Chicago, IL, USA. The alkaline phosphatase (ALP) quantitative detection kit was purchased from the BD Company, Franklin Lakes, NJ, USA. The alizarin red S powder was purchased from the Sigma Aldrich Corporation (Saint Louis, MO, USA). The mouse-anti-human TG2 and Caspase-3 monoclonal antibodies were obtained from the Santa Cruz Biotechnology Corporation (Santa Cruz, CA, USA).

The preparation of the solution for the calcium salt precipitation experiment was as follows: 50 mg/ml vitamin C stock solution, 100 mM β-glycerophosphate stock solution, 0.25% glutaraldehyde and 2% alizarin red S (pH 4.2).

The super-clean work bench (SW-CJ-2FD, Suzhou Antai Air Technology Co., Ltd), CO_2 incubator (6500, Thermo Fisher Corporation, Waltham, MA, USA), inverted phase contrast microscope (Nikon Corporation, Tokyo, Japan), cell culture plate (Corning Corporation, New York, NY, USA), low temperature refrigerator -80°C (Sanyo Corporation, Hokkaido, Japan).

Experimental Methods

Cell culturing was performed as follows: a routine cell thaw followed by inoculation and subculture. The C3H10T1/2 cells were cultured in basal medium eagle (BME) culture medium (used to stimulate osteogenic differentiation). Differentiation and grouping were induced (inoculate the C3H10T1/2 cells into 24-well cell culture plate by 3×10^4/pore, used proper titer of AdBMP9 to infect the cells (infection rate was 30%). After the cell culture comes to logarithmic phase (fusion degree was about 30%), change for BME culture medium after 8 hours and consecutively culture for 3-20 days. Place the cells into 37°C anoxic incubator with 5% CO_2 and 95% N_2 for culturing and build the anoxic model. Established groups were experimental or observation group and control group. The control group was treated with pure BMP-9 induction and the observation group was treated with BMP-9 combined with TG2 induction under oxygen deficient condition.

Observation Index

Improved secreted alkaline phosphatase (SEAP) chemiluminescence method and histochemical staining method were used to detect the expression of alkaline phosphatase (ALP). The specifics are as follows: inoculated the C3H10T1/2 cells into 24-well plate by 3×10^4/pore, induced differentiation, discarded the culture medium. Split and decompose the cells. Supernatant and the ALP substrate were removed following centrifugation. Then, blended the cells with Lupo buffer solution, and the cells were placed in a dark spot at room temperature. The ALP activity was detected by chemiluminescence method. This was followed by the application of the alizarin red S stain to detect calcium salt precipitation. The following procedure was used to conduct the experiment. Inoculated the C3H10T1/2 cells into 24-well plate by 3×10^4/pore and induced differentiation. Then, discarded the culture medium and washed the cells with PBS. Cells were fixed with 2.5% glutaraldehyde and washed with deionized water. Next, 0.4% alizarin red was added to dye, discarded the dye liquid and ended the reaction by washing with double distilled water. After the procedure was completed, the imaging results were observed using a light microscope. Expression of the caspase-3 protein was detected using the Western blot method. The cells were resuspended in the cell lysis buffer, centrifuged, and the

cell protein extract was placed into the microtiter plate pore. Each pore was mixed with 100 μL reaction buffer solution, Ac-DEVD-pNa with concentration of 2 mmol/L to detect caspase-3 and measure absorbency by microplate spectrophotometer at 405 nm. Then, the paranitroaniline was applied to obtain a standard curve and define the concentration of the end product. Flow cytometry was used to detect the cell cycle stage of the cells. Used pancreatin to digest and absorb the cells, followed by washing with PBS, and fixed with 70% cold ethanol. Then, 500 μl of PBS solution composed of 100 μg/ml RNase was added, followed by 1 ml PBS solution composed of 20 μg/ml propidium iodide (PI). Staining was carried out in the dark, and applied BD. LSR flow cytometry detected fluorescence intensity (excitation wavelength of 488 mm). Then, the volume of DNA in the cell cycle was analyzed with the MODFIT2.0 software. Trypan blue exclusion was used to count the living cells and monitor cell proliferation. The C3H10T1/2 cells were removed to suspend the cells. Then, the cell density was adjusted and the cells were inoculated into each T-25 culture bottles (1.2×10^5 cells/bottle). Next, the same amount of culture solution was added in each bottle, and trypan blue staining was used to count the number of living cells. The experiment was repeated three times to obtain an average value.

Statistical Analysis

The SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA) was applied to analyze the data. Measurement data was presented using ± standard deviation; *t*-test was applied in comparisons between the groups. $p < 0.05$ was considered statistically significant.

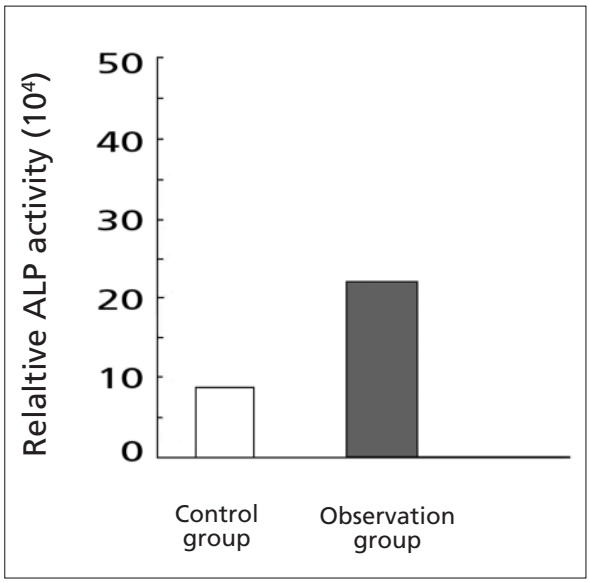

Figure 1. Comparison on the expression level of early ALP.

Results

Comparison of the ALP Expression Level and Precipitation of the Calcium Salt

The level of ALP expression in the experimental group was higher than in the control group ($p < 0.05$) as shown by the precipitation of the calcium salt (Figures 1, 2).

Comparison of the Expression Level of Caspase-3 Protein

The levels of expression of both early and advanced caspase-3 protein in the experimental group were significantly ($p < 0.05$) lower than those in the control group (Table I).

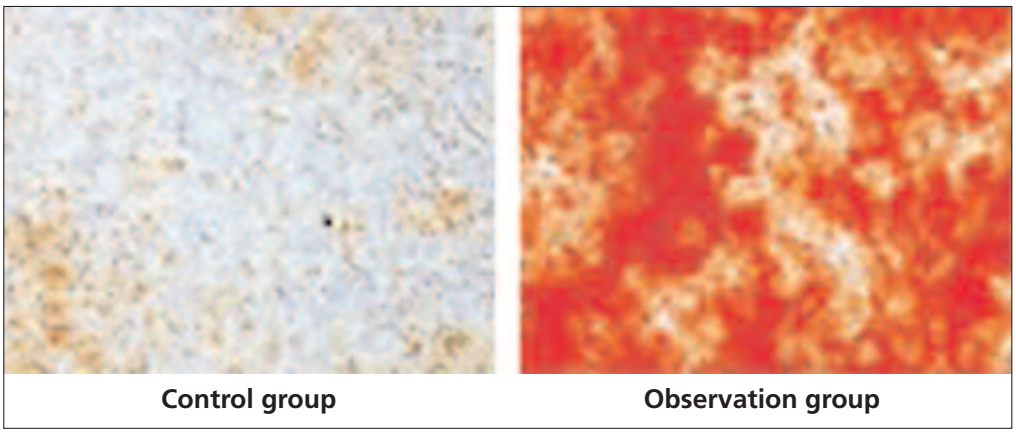

Figure 2. Comparison on the precipitation level of advanced calcium sale.

Table I. Comparison on the expression level of the caspase-3 protein.

Group	Early caspase-3 level	Advanced caspase-3 level
Control group	62.4 ± 5.6	42.3 ± 4.9
Observation group	41.8 ± 3.8	26.9 ± 3.2
t	3.529	3.876
p	0.037	0.034

Comparison on Cell Cycle and the Number of Dividing Cell

The highest fraction of cells in the experimental group was in the M-phase while in the control group cells was mostly in the interphase. In addition, the number of cells in the experimental group was significantly higher ($p < 0.05$) than in the control group (Figures 3, 4).

Discussion

The correlation between proliferation and differentiation of the cells was analysed. The balance could guarantee normal, healthy growth while the imbalance might result in various types of lesions, and even tumors. The BMP-9 could not induce osteogenic effects in human osteosarcoma cells. But, it could promote proliferation and growth of the tumor, which indicates that the occurrence and development of the sarcoma and osteoma are closely related to the osteogenesis differentiation defects of the cells[11]. The BMPs transfers the signals mainly through typical BMPs-Receptor-Smad signal transduction and thus promote osteogenesis[19]. BMPs, when combined with TGFβ II and TGFβ I receptors that are equipped with serine/threonine kinase activity form a compound and activate the Type I receptor. The Type I receptor recognizes the corresponding R-Smads inside the cells, such as Smad1, Smad5, Smad8, and phosphorylates them. The phosphorylated R-Smads then combine with the Smad4 and assist in the transcription of specific target genes and finally induce effects[14,20]. Although BMP9 could induce osteogenic differentiation, its osteogenesis and osteanagenesis effects still have some shortcomings. It might be because the BMP factors could promote collagen synthesis[21]. The TG2 is a special member of the glutamine transaminase family. Studies have confirmed that TG2 is involved in the regulation process of multiple cell apoptosis[22]. Under anoxia, high- level ROS would trigger Ca^{2+} to enter the cells, which would result in the en-

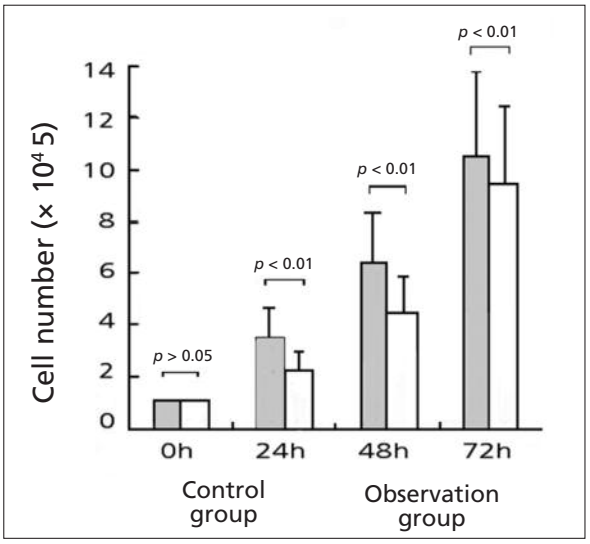

Figure 3. Comparison on the number of dividing cells.

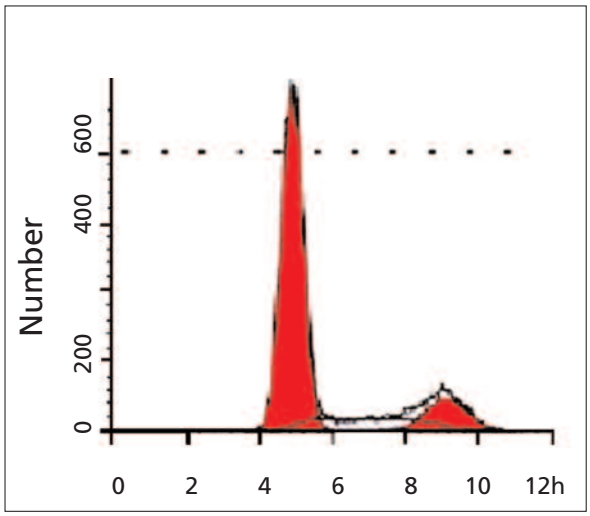

Figure 4. Comparison on the cell cycle.

hanced enzyme activity of TG2 leading to cell apoptosis[23,24]. Recent studies[25-27] have confirmed that inducing the TG2 expression could promote the survival of renal carcinoma cells. The mutated TG2 (R580A) inside the cell nucleus could offset the apoptosis promoting the effect of TG2 on the cytoplasm. These results indicate that the TG2 could not only promote apoptosis but also resist apoptosis. Caspase-3 is an important effector in caspases family. Under anaerobic environment, the cross-linked polymer that results from the TG2 activation inside the cytoplasm could combine with the caspases-3 and form an insoluble component, therefore, inhibiting the caspase-3 activity and reducing the anoxia-induced apoptosis[28,29]. ALP was the classic indicator of early osteoblastic differentiation while calcium salt precipitation was the classic sign of advanced osteoblastic differentiation. In this study, ALP and calcium salt precipitation were used as the basic indicators. The changes in the cell cycle transformation and the changes in the cell proliferation were observed and the relationship between cell proliferation and the osteogenesis was analyzed. From the results of this study, it was found that: the early expression levels of ALP in the observation group were significantly higher than that in the control group. The precipitation of the calcium salt levels in the experimental group was significantly superior to that in the control group. The expression levels of both the early and advanced caspase-3 protein in the experimental group were significantly lower than those in the control group. The observation group was led by M phase while the control group was led by interphase and the cell number in the observation group was greater than that in the control group. The differences had statistical significance.

Conclusions

The BMP-9 in combination with TG2 was better to inducing the mice MSCs C3H10T1/2 osteogenesis, suggesting interaction between MAP-9 and TG2 and the involvement of TG2 in osteogenesis. However, further studies are needed to elucidate the exact mechanism of MAP-9/TG2 interactions.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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