Effects of rutin on osteoblast MC3T3-E1 differentiation, ALP activity and Runx2 protein expression

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As a flavonoid, rutin has been found to have a wide range of biological functions, such as resisting inflammation and oxidation, and preventing cerebral hemorrhage and hypertension. It has been found to play an important role in osteoporosis and other orthopedic diseases in recent years. MC3T3-E1 cells were randomly divided into a control group, a rutin-1 group (0.01 mmol/L), a rutin-2 group (0.05 mmol/L) and a rutin-3 group (0.1 mmol/L). Osteogenic differentiation of cells was induced by osteogenic induction fluid. The control group was treated with the maximum dose of drug solvent. 2~3 days later, the solvent was replaced with fresh osteogenic induction fluid containing rutin. After a certain period of routine culture, the cells were collected for subsequent experiments. The expression of Runx2 gene in cells in all groups was detected by Real-time PCR; the expression of Runx2 protein was detected by Western blot and immunocytochemistry (IHC); the activity of ALP was detected by reagent kit method; osteogenic differentiation was analyzed by alizarin red staining. The results of Real-time PCR showed that, compared with the control group, the treatment of cells with rutin can significantly increase the expression of Runx2 gene (p<0.05); the higher the concentration, the higher the expression of Runx2 gene, and significant differences were found among groups in which different concentrations were used (p<0.05); the results of Western blot and IHC showed that the expression trend of Runx2 protein in each group was consistent with PCR results. In drug treatment groups, the activity of ALP was significantly higher than that in the control group (p<0.05); there were significant differences among groups in which different concentrations were used (p<0.05). The results of alizarin red staining showed that calcified nodules were formed in all groups and that the area of calcified nodules formed in groups treated with rutin was greater than that in the control group; the greater the concentration, the larger the area. Rutin can promote osteoblastic differentiation; and the greater the concentration, the more effective it is.

Key words: xRutin; Runx2; ALP; osteogenic differentiation.

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Introduction

Rutin is a kind of flavonoid mostly from rutin leaves, tobacco leaves and *Sophora japonica* buds, etc. It is widely used and has such functions as resisting inflammation and oxidation and maintaining vascular resistance. It is clinically often used to treat hypertension, prevent vascular sclerosis, cerebral hemorrhage and other diseases. In this study, mouse osteoblast precursor cells, MC3T3-E1 cells, were used as study subjects to explore the effects of different concentrations of rutin on the expression of Runx2 protein and the activity of ALP during MC3T3-E1 differentiation. MC3T3-E1 cells biological functions are close to those of primary cultured osteoblasts. Therefore, they are extensively used to study the differentiation, proliferation and molecular mechanism of osteoblasts.

During bone development, the expression of Runt family associated transcription factor 2 (Runx2) is the beginning of osteoblast differentiation. Runx2 can initiate and activate the differentiation of bone narrow stromal stem cells into osteoblasts. Meanwhile, it can upregulate the expression of many osteogenic genes. It is a key marker in bone formation. In addition to playing a biological role in osteoblasts, Runx2 is highly expressed in hypertrophic chondrocytes and promotes chondrocyte hypertrophy and endochondral ossification. Runx2 also promotes osteoclast differentiation by inducing the nuclear factor κB receptor activator ligand (RANKL) and inhibiting the osteopontin (OPN) and osteosialin (BSP).

Alkaline phosphatase (ALP) is an isoenzyme widely existing in liver, bone, placenta and other tissues. It is clinically used for the diagnosis and differentiation of rickets, malignant tumor bone metastasis, hepatobiliary diseases and other diseases. During bone formation, ALP can hydrolyze phosphate and pyrophosphate to eliminate their inhibitory effect on bone formation, which is beneficial to further bone formation. The activity of ALP can indicate the degree of differentiation of osteoblasts and is used as one of the indicators to evaluate the mineralization ability of osteoblasts.

Materials and Methods

**Cell culture**

Mouse embryonic osteoblast precursor cells, MC3T3-E1 cells, were purchased from Shanghai Cell Bank, Chinese Academy of Sciences. Routine culture was performed with the α-MEM medium containing 10% FBS and 1% P/S (PM150421, Procell, Wuhan, China) at 37°C and 5% CO₂. The state of MC3T3-E1 cells was adjusted. MC3T3-E1 cells in logarithmic phase were randomly divided into a control group, a rutin-1 group (0.01 mmol/L), a rutin-2 group (0.05 mmol/L) and a rutin-3 group (0.1 mmol/L). Osteogenic differentiation of cells was induced by osteogenic induction fluid. The control group was treated with the maximum dose of drug solvent; 2–3 days later, the solvent was replaced with fresh osteogenic induction fluid containing rutin. After a certain period of routine culture, the cells were collected for subsequent experiments. Rutin was purchased from Solarbio Technology Co., Ltd. (SR8250; Beijing, China).

**Real-time PCR**

Cells were collected from all groups after 3 days of treatment with rutin. The total RNA in cell samples from each group was extracted according to the instructions for TRIpure extraction kit (RP1001; BioTek, Beijing, China). The ultraviolet spectrophotometer NanoDrop-2000 (ThermoFisher Scientific, Waltham, MA, USA) was used to determine the RNA concentration in each sample. Real-time PCR (Exicycler 96, BIONEER, Republic of Korea) was used; and super M-MLV reverse transcriptase (PR6502; BioTeke, Beijing, China) was used for reverse transcription. Real-time fluorescence quantitative PCR reaction was carried out by adding 2×Power Taq PCR MasterMix (PR1702; BioTeke), SYBR Green 1 (SY1020; Solarbio), cDNA template and upstream and downstream primers. Upon the completion of the reaction, β-actin was used as a reference. The formula 2^−ΔΔCt was used to calculate the relative expression level of mRNA of Runx2 gene. See Table 1 for primer sequence. It is synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

**Western blot**

The cells in all groups were treated with rutin for 3 days. The treated cells were split with the RIPA lysis buffer (P0100; Solarbio) on the icebox for 5 min; then the cells were centrifuged at 12,000 rpm and 4°C for 10 min. The supernatant was separated to obtain protein extract. BCA protein assay kit (WLA004, Wanleibo, Shenyang, China) was used for quantitative analysis of the total protein (TP) extracted to get protein concentration. The electrophoresis apparatus was assembled and polyacrylamide gel prepared. 8% SDS-PAGE was used for electrophoresis. After electrophoresis, the protein bands separated were transferred to the PVDF membrane (IPVH00010; Millipore, Burlington, MA, USA). The PVDF membrane was sealed with 5% skim milk; then Runx2 primary antibody (diluted by 1:500; WL03358; Wanleibo) was added. The PVDF membrane was incubated at 4°C overnight. Upon the completion of incubation, the PVDF membrane was washed with TBST. The horseradish peroxidase (HRP)-labeled sheep anti-rabbit IgG (diluted by 1:5000; SE134; Solarbio) was added for incubation. The reference β-actin was incubated by the same experimental procedures. Finally, ECL luminous fluid (PE0010; Solarbio) was added for exposure in a darkroom. The film was scanned. The gel image processing system (the software Gel-Pro-Analyzer) was used to analyze the optical density of the target band.

**Immunocytochemistry (IHC)**

After 3 days of treatment with rutin, the cells treated in each group were made into cell slides. Four percent paraformaldehyde solution was used for fixation for 15 min. Next, 0.1% TritonX-100 was added for incubation at ambient temperature for 20 min and then discarded. 3% H₂O₂ was added for incubation. The serum was added for sealing for 15 min. The serum was discarded, the Runx2 primary antibody diluted with PBS (diluted by 1:500; WL03358; BioTeke) was added for incubation at room temperature for 20 min. After incubation, the slides were rinsed with PBS three times for 5 min. After the last rinse, the slides were incubated with goat anti-rabbit IgG (diluted by 1:1000; SE134; Solarbio) for 20 min. The slides were washed and added for incubation. The reference β-actin was incubated by the same experimental procedures. Finally, the slides were incubated with the secondary antibody (diluted by 1:500; SE134; Solarbio) for 20 min. The slides were washed and added for incubation. The reference β-actin was incubated by the same experimental procedures. Finally, the slides were incubated with the secondary antibody (diluted by 1:2000; SE134; Solarbio) for 20 min. Then, the slides were rinsed with PBS three times for 5 min. The slides were incubated with DAB solution for 3 min, then washed with PBS and with distilled water for 3 min. The slides were mounted with coverslips and covered with neutral gum. The slides were observed under an optical microscope.

Table 1. Primer sequence.

| Name of primer | Former primer (5’–3’) | Back primer (5’–3’) |
|----------------|-----------------------|--------------------|
| Runx2          | GCACACATCAATCTCCTAC   | TTTCGTCAGGTCGACAC  |
| β-actin        | CTGTCCCCATCTACGAGGCTAT| TTTGACTCATCGGACAGATTC |

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Effects of rutin on ALP activity in cells

The ALP activity was detected with ALP activity detection kit after the cells in all groups were treated with rutin for 5 days. It was found that, compared with the control group, the ALP activity of all treatment groups was significantly improved, and the greater the concentration, the higher the ALP activity (p<0.05); and there were significant differences in the ALP activity among different concentration groups (p<0.05) (Figure 3).

Detection of osteogenic differentiation of cells by alizarin red staining

The differentiation in cells was analyzed by alizarin red staining after 14 days of treatment with rutin. The results showed that calcified nodules were formed in all groups and that the area of calcified nodules formed in groups treated with rutin was greater than that in the control group; the greater the concentration of rutin, the larger the more obvious the nodules (Figure 4).

Discussion

Rutin is a flavonoid found in many plants, has a wide range of biological activities, and can be used to resist oxidation and inflammation, protect the nerve and liver, and prevent cerebral hemorrhage. In addition to some diseases that have been known and clinically treated with rutin, the role of rutin in other diseases has also been gradually taken seriously by people. Previous studies showed that rutin has important potential in the prevention and treatment of diabetes. The effects of rutin in orthopedic diseases have also been gradually known by people. Osteoporosis is a chronic disease; the main cause is that the bone has lost its mineralization quality and mechanical flexibility. Since the currently used anti-osteoporosis agents have some efficacy but also some side effects, including an increased risk of blood clots and cancer, phytochemicals may be a safer and more effective option. The

Effects of rutin on expression of Runx2 gene in cells

After 3 days of treatment of MC3T3-E1 cells with different concentrations of rutin, the expression of Runx2 gene was detected by PCR. The results showed that, compared with the control group, 3 concentrations of rutin could significantly increase the expression quantity of Runx2 gene, and there were significant differences (p<0.05). The relation between different concentrations of rutin was compared; and it was found that the greater the concentration, the higher the expression quantity of Runx2 gene. There were highly significant differences among the 3 concentrations (p<0.05) (Figure 1).

Effects of rutin on expression of Runx2 protein in cells

After the treatment of cells in all groups with rutin for 3 days, the expression of Runx2 protein was detected by Western blot (WB) and IHC, respectively. WB results showed that rutin can significantly increase the expression level of Runx2 protein, and the difference was statistically significant; with the increase of rutin concentration. The expression level of Runx2 protein also got higher and higher, and there were significant differences (p<0.05). The IHC results showed that, there was no significant difference between the low concentration group and the control group. The comparative results among other groups were consistent with those of WB (Figure 2 A,B).
study on human osteosarcoma cells, SAOS-2 cells, showed that rutin can increase the expression of osteocyte and osteoblast-related genes and decrease the expression of Runx inhibitor and osteoclast gene. The study on the effects of rutin on ALP activity demonstrated that rutin can improve ALP activity and decrease the activity of acid phosphatase, a marker for osteoporosis. Therefore, rutin can promote the proliferation of osteocyte and act as an ossification marker. The results of this study are consistent with those of our study, proving that rutin can be used as an effective drug for future treatment of orthopedic diseases. Xiao et al. explored the mechanism of action of rutin in osteoporosis and found by analyzing by multiple experimental methods, including Micro-CT, Western-Blotting, Real-time PCR, transmission electron microscope and alizarin red staining, that rutin can regulate FNC1D1 level and autophagy through the Akt/mTOR signaling pathway, which provides a new strategy for the treatment of osteoporosis. Rutin can also protect human periodontal ligament stem cells (HPDLSCs) from the TNF-α-induced osteogenic differentiation damage in an inflammatory environment, and is expected to be a new candidate drug for the treatment of periodontitis bone defects.

The formation and reconstruction of bone is a complicated process, including osteoblasts forming new bone and osteoclasts absorbing old bone to maintain a dynamic balance. Runx2, also known as core-binding factor al, is one of the important members of the Runt family. The other two members are Run1 and Runx3. Some studies have shown that, in the early stage of osteoblast differentiation, Runx2 gene can trigger the synthesis of bone matrix protein and provide a great number of immature osteoblasts. It can be used as a specific marker gene to indicate osteoblast differentiation and bone formation. Moreover, Runx2 gene activates the transcription and expression of bone sialoprotein, osteopontin and type 1 collagen gene by binding to cis-acting elements of osteoblasts. The molecular osteoprotegerin (OPG) produced by osteoblasts plays an important role in the formation of osteoclasts; OPG can not only inhibit the formation of osteoclast-like cells in

Figure 2. Expression of Runx2 protein in cells after treatment with rutin. A) Western blot; B) Immunohistochemistry.
vivo and in vitro but also absorb bone. The sequence cloning of human OPG showed that there is a binding site for OPG in Runx2 gene sequence, indicating that Runx2 can regulate OPG expression.9,20 Runx2 gene is highly expressed in multiple myeloma cells and is one of the major drivers of myeloma progression. Lowering the abundance of Runx2 by regulating the upstream genes of Runx2 can slow down the progression multiple myeloma.21 The results are consistent with ours. With the deepening of study and the understanding of the richer functions and mechanism of action of Runx2 gene, it will certainly provide a detailed theoretical basis and practical significance for the treatment of bone-related diseases.

ALP is a specific enzyme secreted by osteoblasts. Its activity directly reflects the degree of the differentiation of osteoblasts. Zhang et al. confirmed that ALP was highly expressed in both in vitro osteogenic differentiation and in vivo bone formation.22 Similar to rutin, astragalin is also a bioactive flavonoid mostly from medicinal plant. After the treatment of MC3T3-E1 cells with astragalin, high expression of ALP was also detected and significantly increased; besides, the expression level of Runx2 was also higher than that in the control group. The results are consistent with those of this study.23 It is believed that, with the deepening of study, rutin will play a better role in the treatment of orthopedic diseases, with great significance for their cure.

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