Improvement of the ability of bone repair by reducing the aging degree of mesenchymal stem cell population by Senolytic drugs

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Abstract. In order to study the effects of Senolytic drugs dasatinib and quercetin on the aging of mesenchymal stem cells (MSCs) and explore the application of young MSCs in repairing bone defects, the cultured and identified MSCs were treated with dasatinib and quercetin, counting the number of passage times, and the senescence of cells was evaluated by high-content screening microscopy. The 4th-passage MSCs rejuvenated with Senolytic drugs were transplanted into bone defect model rabbits, and X-ray examination was performed 12 weeks after surgery. The results showed that Senolytic drugs reduced the senility of MSCs and improved the ability of bone repair.

1 Introduction

Mesenchymal stem cells (MSCs) are stem cells with multidirectional differentiation potential and are needed in large quantities in regenerative medicine [1]. However, MSCs amplification will cause senescence, reduce differentiation ability, and limit its clinical application value. Therefore, this study aimed to reduce the aging of MSCs and improve the ability of osteogenic differentiation. We used the newly discovered Senolytic drugs dasatinib and quercetin to "rejuvenate" the MSCs population, and to test the influence of Senolytic drugs on the passage number of MSCs population. The effect of Senolytic drugs on the senile degree of MSCs was evaluated by counting of cell passage times and high-content screening microscopy. After the MSCs were treated with Senolytic drugs, in order to determine the osteogenic ability of the cells in vivo, the cells were transplanted into the bone defect model rabbits, and the ability of MSCs to repair bone defects was analyzed by X-ray.

2 Methods

2.1 Acquisition, culture and identification of MSCs

In this study, 17 female adult New Zealand white rabbits (2.92±0.26kg, 7-8 months of age) were purchased from the Experimental Animal Center of the Third Xiangya Hospital of Central South University and reared in the standardized animal culture room of ChangSha Medical University. Five rabbits were used to obtain MSCs: Adipogenic MSCs were isolated from the inguinal fat pad of rabbits by mononuclear cell isolation fluid. The separated cells were cultivated in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco, USA) containing 10% new-born bovine serum (Invitrogen, USA), penicillin (100 U/ml) and streptomycin (100 g/ml) at 37°C, 5%CO2 and 95% humidity. After 24 h, the unattached cells were removed by rinsing with PBS. MSCs were identified by flow cytometry using surface antigens of CD44, CD45, CD90 and CD105. At present, this part of the experiment has been completed and the technology is mature.

2.2 Count of cell passage times

Replicative aging cell model was prepared by continuous culture with 70% coverage of MSCs after cell fusing as the passage standard. The difference in the number of MSCs passage between the experimental group treated with Senolytic drugs (dasatinib 5µM, quercetin 5µM) and the blank control group was compared. The experiment was repeated for 8 times.

2.3 Evaluation by high-content screening microscopy technology

The cells of the 4th and 8th passage from each group were inoculated in a 6-well plate at a density of 1×10⁶/mL. After 48 h of attachment and proliferation, the cells were fixed with 4% paraformaldehyde. After 0.1%Triton X-100 treatment, the cytoplasm was stained with 1 μg/mL Mask Deep Red. The nuclei were stained with 0.125 μg/mL DAPI. The images of the nucleus and cytoplasm were generated by signal acquisition at 385nm and 630nm, respectively. Each sample was analyzed three times and averaged to correct the data. The cell area sizes were calculated using the Thermo Scientific software of the high-content screening microscope. The experiment was repeated for 6 times.

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2.4 Measurement the repair effect of bone injury

MSCs were differentiated and cultured in osteogenic medium containing β-sodium glycerophosphate, dexamethasone and ascorbic acid, and at the same time, dasatinib(5µM) and quercetin(5µM) were used to promote cell population rejuvenation. Twelve rabbits were divided into experimental group, negative control group and blank control group to compare the effect of osteogenesis in vivo (n=4). After anesthetizing the experimental animals, the proximal cortical bone of the palmar side of the radius was excised in rabbits with a size of about 15mm×6mm, and the model of large bone defect of the radius was artificially constructed.

The conventional cultured 4th-passage MSCs attached 10% β-TCP/gelatine scaffold were used as the negative control group, and the same scaffolds attached by the 4th-passage MSCs treated by the Senolytic drug were used as the experimental group and transplanted to the bone defect model animals, respectively, while those without transplantation were used as the blank control group. X-ray examination was performed 12 weeks after surgery to compare the repair effect of bone injury.

2.5 Statistical analysis

All values were expressed as the means ± SD. Differences among several groups were determined by one-way ANOVA; comparisons between groups were performed by an LSD t-test using SPSS software (version 9.0). P<0.05 was considered statistically significant.

3 Results

3.1 Number of passage

The limit to the number of normal cell divisions is relatively fixed. So if the cells is treated, the number of passages increase significantly, indicating that the cell population is relatively young. The number of passages is a simple and unambiguous indicator of cell aging. Through continuous culture, the number of cell passage in the experimental group of dasatinib and quercetin was significantly different from that in the control group. The number of passage in the control group without dasatinib and quercetin was (14±1.2) times. That was, after dividing 14 times, the cell doubling time was significantly prolonged and cell proliferation was slowed down, so it was difficult to reach 70% cell coverage. In the experimental group, the passage times were (17±0.9) times. That was, the average times of cell division was 17 before the above phenomenon gradually appeared. The difference between the two groups was statistically significant (P<0.05). (Fig. 1.)

3.2 Cell area

The sample area of control group in the 4th passage was (3022±420) μm², and the homogeneity was good. The area of most cells in the 8th passage was significantly increased, but there were a few small cells, and the area difference was larger. The cell area calculated by software was (8955±1308) μm². Compared with the control group, the sample area in the 4th passage of experimental group was (2886±602) μm², and there was no significant difference (P>0.05). The area of most cells in the 8th passage of experimental group was (7020±454) μm², and the average area was smaller than that in the control group (P<0.05), and the homogeneity was better. (Fig. 2.)

Considering that cell confluence was an indicator of passage, the increase of cell size reflected the decrease of cell number in each passage group, and the prolongation of culture time indicated the decrease of cell proliferation ability. Also in the 8th passage, the cell area of the experimental group was smaller than that of the control group, indicating more cells and better cell proliferation ability.
3.3 Comparison of osteogenic effects

Twelve weeks after the operation, the rabbits in the blank control group were relatively emaciated and inactive. Although the wound was healed, movement of the limbs on the side of the bone defect was limited. The rabbits of the negative control group and experimental group not only had healed wounds, but also moved their limbs as usual, which was not significantly different from that of healthy rabbits.

At 12 weeks after operation, the broken ends in the blank group were closed and slightly enlarged, and the bone repair was poor, indicating that the large bone defect was beyond the self-repair ability of the experimental rabbits. The negative control group was transplanted with untreated MSCs. The bone defect area was reduced, the bone defect area was blurred, and the new callus density was increased. In the experimental group, the defect area was basically healed, the density was slightly lower than the primary bone, and the bone defect area was not obvious. (Fig. 3.)

Fig. 3. Bone repair effect of MSCs treated with Senolytic drugs
(1:Blank control group; 2:negative control group; 3:experimental group)

4 Conclusions

Senescence is a normal physiological phenomenon in the development of a living organism. With the aging of the organism, the functions and physiological states of the tissues and organs of the organism will gradually decrease [2-3]. Adult stem cells are no exception. In general, MSCs cannot proliferate and differentiate indefinitely, and signs of aging will appear after a certain number of generations of replication [4], namely replicative aging. Both the regulatory function of MSCs and the function of differentiation to target cells will gradually decline with the occurrence of replicative senescence. The stability of MSCs will be weakened, leading to the weakened proliferation ability and seriously affecting the function and growth state of the body [5]. In 2015, a major breakthrough in anti-aging research appeared. A new class of drugs have been developed by a research team from the Scripps Research Institute (TSRI) in the United States, that significantly slow the aging process in animal models - relieving debilitating symptoms, improving heart function and extending healthy life [6]. Scientists called the new drug "Senolytic." The new study was published in the August issue of the journal Aging Cell. The idea of developing such Senolytic drugs is completely new. It no longer focuses on the analysis of the aging mechanism of a single cell, but can be "rejuvenated" from the cell population by removing senescent cells. Of the Senolytic drugs, the first to be found to be effective were dasatinib and quercetin [7-9]. The drugs extend the "healthy life span" of mice by clearing away senile cells. The preliminary experiment of quercetin and dasatinib single drug intervention was carried out at the beginning of the study, and the effect of the drug was not very good. Therefore, according to the suggestion of Weivoda
research group, dasatinib and quercetin were combined for intermittent administration, and the significant effect was found to take up to 8 weeks at the earliest. These drugs are considered to be the most clinically promising treatment at present [10]. But experiments are mainly conducted on the whole animal, and there are very few experiments at the cell level.

In our study, we isolated and cultured umbilical cord blood and adipogenic MSCs in the early stage, and induced differentiation of MSCs into osteoblasts to explore the ability of MSCs to repair bone defects. This research also encountered the same dilemma: in order to complete on experimental animal bone defect repair, a lot of seed cells were needed, so cell amplification were repeated, while after frequent proliferation of MSCs showed the aging trend, division and differentiation ability significantly reduced, then cellular osteogenesis ability decreased obviously after cultivating more times. If the amplification results in cell senescence and decreased cell quality, the clinical and practical value of MSCs is limited undoubtedly. Therefore, our research group took delaying the senescence of MSCs group as the breakthrough goal to study whether Senolytic drugs can clear senescent cells in MSCs group, improve the proportion of young cells, and make MSCs overall younger.

In this study, referring to other research groups [1,11-13], we selected a certain concentration of dasatinib and quercetin combined to act on mesenchymal stem cells to test whether classical Senolytic drugs can improve the quality of seed cells. The precise calculation of cell area was carried out using a high-content screening microscope, which is an efficient automatic screening system for fluorescence image acquisition and quantitative analysis. In previous studies [14], we confirmed that changes in cell area are closely related to the expression of senescence markers, and that the increase in area caused by senescence can be easily detected with good repeatability. This noninvasive microscopic technique can be used in routine stem cell culture to determine the senescence of cultured cells. Finally, through the repair of animal bone defect model, we compared whether the young MSCs could better proliferate and differentiate. The experimental results were encouraging. By counting the numbers of cell passage and accurate measurement of cell area, Senolytic drugs are found to reduce the aging degree of MSCs to a certain extent and improve the bone repair ability of MSCs through the X-ray detection. This provides an effective solution to the problem of quality degradation caused by the proliferation of stem cells during stem cell production.

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