Research Article

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Paracrine study of adipose tissue-derived mesenchymal stem cells (ADMSCs) in a self-assembling nano-polypeptide hydrogel environment

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Abstract: To research the paracrine role of adipose tissue-derived mesenchymal stem cells (ADMSCs) in promoting angiogenesis under the three-dimensional culture conditions consisting of a functionalized self-assembling peptide nanofiber hydrogel. ADMSCs were isolated, extracted, and then identified. Three kinds of peptides (RADAI-16, RGD, and KLT) were prepared, and a functionalized self-assembling peptide nanofiber hydrogel was produced by mixing RADAI-16, RGD, and KLT in a volume ratio 2:1:1. AFM was used to observe RADAI-16, RGD, KLT, and the functionalized self-assembling peptide nanofiber hydrogel. Then, ADMSCs were cultured under three-dimensional conditions consisting of the peptide nanofiber hydrogel, and AFM was used to observe cell migration. The ADMSCs in the common culture group (37°C, 5% CO₂ cell culture box) and hypoxic culture group (37°C, 10% CO₂, and 1% O₂ hypoxic culture box) acted as controls. ADMSCs were three-dimensionally cultured in situ for 1 day, and then the concentrations of HGF and VEGF in the supernatant were determined by ELISA. Cells were extracted from the peptide nanofiber hydrogel, and HO-1 expression was detected by western blotting. ADMSCs have high expression levels of CD29, CD90, and CD105 and low expression levels of CD34 and CD45. In addition, they can differentiate into adipocytes and osteocytes. The diameters of the fibers of RADAI-16, RGD, KLT, and the functionalized self-assembling peptide hydrogel are 17.34 ± 1.82, 15.50 ± 1.41, 13.77 ± 1.18, and 20.26 ± 1.25 nm, respectively. AFM indicated that cells in the functionalized self-assembling peptide nanofiber hydrogel migrated farther than those in RADAI-16. The concentrations of HGF under common, hypoxic, and three-dimensional culture conditions were 47.31 ± 6.75, 247.86 ± 17.59, and 297.25 ± 17.95 pg/mL, respectively, while the concentrations of VEGF were 218.30 ± 3.03, 267.13 ± 4.27, and 289.14 ± 3.11 pg/mL, respectively. Both HGF and VEGF were expressed more in the presence of the functionalized self-assembling peptide nanofiber hydrogel than in its absence (P < 0.05). Using western blotting, ADMSCs cultured under hypoxic and three-dimensional conditions were found to have high expression levels of HO-1. Culturing ADMSCs under three-dimensional conditions consisting of functionalized self-assembling peptide nanofiber hydrogels can promote their paracrine role in angiogenesis, such as HGF and VEGF, and hypoxia is one of the important elements.

Keywords: human ADMSCs, three-dimensional culture, functionalized self-assembling peptide nanofiber hydrogel, paracrine, hypoxia

1 Introduction

Mesenchymal stem cells (MSCs), namely, adult stem cells with high self-renewing ability and multidirectional differentiation potential from myeloblasts are extensively present in connective tissues and organ mesenchyme in the whole body. They were first found by Friedenstein in marrow-adhering cell culture at the earliest [1], and subsequently, similar MSCs were found in tissues such as
cord blood, peripheral blood, muscle, and fat. By virtue of convenient acquisition, extensive sources, the high number of obtained cells, and minor damage to the donor site [2], adipose tissue-derived mesenchymal stem cells (ADMSC) have rapidly become widely accepted seed cells in tissue engineering.

Cells are in a three-dimensional microenvironment in vivo and are influenced by physical signals and bioactive signals, while it is difficult to provide a three-dimensional environment for ordinary cultures. In recent years, nanopolypeptide materials have made progress as biological scaffolds for three-dimensional cell culture. Liu et al. used a functional self-assembling nano-polypeptide hydrogel as a scaffold to culture ADMSCs and found that the expression of paracrine cytokines would increase during three-dimensional ADMSC culture conditions [3]; however, the reasons for this effect were not sufficiently explained. This experiment aims to explore the influencing factors of increasing the expression of ADMSC paracrine angiogenic growth factors under three-dimensional culture conditions in the presence of a functional self-assembling nano-polypeptide hydrogel.

2 Materials and methods

2.1 Materials

The following materials were used: DMEM/F12 (Gibco Corporation); fetal bovine serum (FBS, Gibco Corporation); double antibody (Gibco Corporation); pancreatin; edetic acid (EDTA, HyClone Corporation); I-type collagenase (Sigma Corporation); phosphate buffer solution (PBS, HyClone Corporation); ADMSC osteogenic differentiation induction solution (US Cyagen Corporation); ADMSC adipogenic differentiation induction solution (US Cyagen Corporation); oil red O dye liquor (US Cyagen Corporation); alizarin red S dye liquor (US Cyagen Corporation); alkaline phosphatase staining and Red S and alkaline phosphatase staining were also fused to 60–70%, the culture solution was removed, and the cells were washed. A total of 2 mL of osteoinductive differentiation and Alizarin Red S and alkaline phosphatase staining were also implemented.

2.2 Method

2.2.1 Separation and culture of ADMSC

The animal procedures and human participation/tissues in this study were carried out in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals, with approval from the Animal Ethics Committee of Shandong Academy of Medical Sciences.

Isolated fresh fat samples from patients with benign diseases (patients signed an informed consent form before operation) were transferred onto an ultraclean worktable within 30 min, megascopic blood vessels and connective tissues were washed and removed, I-type collagenase (m/v 0.1%) was added after the fats were cut into pieces (<1 mm³), and the samples were centrifuged at 1,500 rpm for 10 min after oscillation and digestion in a 37°C thermostatic water bath. Sediments at the substratum were suspended using 10% FBS complete culture solution (10% FBS, 1% 100 µg/mL penicillin, and 100 µg/mL streptomycin) and were filtered by passing through a 100-mesh cell screen, and a complete culture solution was added until 5 mL. Then, the sediments were placed into a 25 cm² cell incubator. The solution was replaced for the first time after 48 h, and then it was replaced once every 2–3 days. After primary cells were coated to approximately 90% density at the bottom, subculturing was carried out.

2.2.2 ADMSC osteoinductive differentiation and Alizarin Red S and alkaline phosphatase staining

Cell density was adjusted to $2 \times 10^4$/mL, and 2 mL of the cell suspension was inoculated into a six-well plate precoated with 0.1% gelatin. When the cell density was fused to 60–70%, the culture solution was removed, and the cells were washed. A total of 2 mL of osteoinductive differentiation complete culture solution was added to adult MSCs, the solution was replaced once every 3 days, and observation was conducted after 4 weeks. Alizarin staining and alkaline phosphatase staining were also implemented.

2.2.3 ADMSC adipogenic differentiation and oil red O staining

The cell density was adjusted to $2 \times 10^4$/mL, and 2 mL of the cell suspension was added to a 6-well plate for culturing. The solution was replaced once every 2–3 days, and the culture medium was removed when the cell
density was 100% or the cells were in the fusion state. Then, 2 mL of ADMSC adipogenesis-induced differentiation solution A was added. Three days later, the solution was removed, 2 mL of ADMSC adipogenesis-induced differentiation solution B was added, and the B solution was replaced by the A solution 24 h later. After 5 days of this alternate culturing, the B solution was continuously used to maintain culturing for 7 days, and the culture solution was removed until the fat droplets became large and round. The cells were washed, and fixation for 30 min using 4% paraformaldehyde and staining with 1 mL of oil red O solution for 30 min were performed. Then, the cells were washed and observed under a microscope.

2.2.4 ADMSC ordinary culture and cell supernatant and protein extraction under anaerobic culture conditions

The cell density was adjusted to 1.0 × 10^5/mL, and 200 µL of cells were placed in a 24-well plate. A total of 200 µL of the complete culture medium was added to each well, and after culturing in a 5% CO_2 cell incubator at 37°C for 24 h, the cell culture supernatant was collected, centrifuged at 3,000 g/min for 10 min in a 4°C environment and preserved in an −80°C refrigerator for standby use. The cell total protein was extracted for the follow-up experiment. The cell supernatant and total protein under anoxic conditions (37°C, 10% CO_2 and 1% O_2, anoxic incubator) were obtained using the same method.

2.2.5 Preparation and detection of polypeptide solution

Ten milligrams each of RADA16-I, RGD, and KLT were dissolved in 1 mL of sterile deionized water in the ultraclean worktable, namely, they were completely dissolved through ultrasonic treatment for 30 min. The polypeptides were placed in a 4°C environment for standby use after sterilization using a 0.22 µm filter membrane. RADA16-I:RGD:KLT were blended in a proportion of 2:1:1 and subjected to ultrasonic blending, and the functional self-assembling polypeptide solution was obtained. After the above four types of polypeptide solutions were diluted 20 times, 5 µL of solutions were diluted and dropped on newly peeled mica sheets. They were gently washed using 100 µL of distilled water after standing for 10 s and then were observed under an atomic force microscope after airing.

2.2.6 Three-dimensional in situ culture of ADMSC and cell supernatant and protein extraction

First, 10% sterile sucrose solution was used to adjust the ADMSC density to 1.0 × 10^6/mL. A total of 20 µL of cell glucose solution was rapidly blended with 100 µL of functional self-assembling polypeptide solution, and the mixture was dropped into a Transwell chamber (the Transwell chamber was preplaced in a 24-well plate with each holder containing 400 µL of the complete culture medium). Then, 200 µL of the complete culture medium was gently dropped along the diagonal direction of the chamber, and the chamber was placed in a 5% CO_2 cell incubator at 37°C. The solution was replaced after 15 min of culturing, and the culturing was continued for another 30 min. The chamber was transferred to a 12-hole incubator with 800 µL of the complete culture medium in each hole. After 1 day, the cell culture supernatants inside and outside the chamber and intracellular proteins in the hydrogel were collected.

2.2.7 Determination of VEGF and HGF concentrations using ELISA

ELISA was used to determine VEGF and HGF concentrations in cell supernatants in the common culture group, hypoxic culture group, and three-dimensional culture group.

2.2.8 Determination of the expression of intracellular heme oxygenase-1 (HO-1) under various conditions using a western blot method

ADMSCs cultured under common culture conditions, hypoxic culture conditions, and three-dimensional culture conditions for 1 day were extracted, and total proteins were obtained after pyrolysis, and protein concentration was determined. Equivalent amounts of proteins were taken and loaded, after which they were subjected to PAGE. After being transferred to polyvinylidene fluoride membranes, they were sealed for 1.5 h, and HO-1 primary antibody (1:1,000) was added and incubated overnight at 4°C. The secondary antibody (1:5,000) was added after washing the membranes, and then they were incubated at room temperature for 2 h, developed using an enhanced chemiluminescent agent, and finally imaged using a gel-imaging system.
2.2.9 Statistical method

Among the experimental results, all data were expressed as the mean ± standard deviation (\( \overline{x} \pm s \)). SPSS20 statistical software was used for data analysis. One-way ANOVA was used for intergroup comparison. \( P < 0.05 \) indicated that a difference was statistically significant.

3 Results

ADMSC primary cells obtained through separation had slow growth and proliferation; they adhered to walls during the growth process and presented a fusiform shape. The cells increased about 7 days later and presented a long fusiform shape, and the cell growth presented a vortex shape with sizes of 30–50 µm (as shown in Figure 1). After osteoinductive differentiation of ADMSC, cells were transformed from the original fusiform shape into triangular and polygonal shapes; particular matters were sedimented in the cells, and sediments increased continuously with time and finally presented a linear shape (as shown in Figure 2). After staining with alizarin red S, calcified nodes were seen in the cells, and black particular minerals were sedimented in cells by alkaline phosphatase staining. The above changes appeared in the control group. After adipogenesis induced differentiation of ADMSCs, cells were transformed from the original fusiform shape into triangular or rectangular shapes, and circular vacuoles structures could be seen in cells with regular morphologies but unequal quantities and sizes. The spherical fat droplets were colored red in cells after oil red O staining with regular morphologies but were of unequal sizes. A similar change did not appear in the control group (as shown in Figure 3).

Figure 1: (a) The shape of the cells is fusiform, and the growth trend is swirled (×40). (b) Further observation of cell morphology and the size of the cell is about 30–50 µm (×200).

Figure 2: (a) After ADMSCs differentiate into osteoblasts, the cell morphology is no longer fusiform (×200). (b) After ADMSCs differentiate into osteoblasts, Alzheimer Red S staining revealed the deposition of red calcium nodules (×40). (c) After ADMSCs differentiate into osteoblasts, alkaline phosphatase staining showed brown calcium nodule precipitation (×40). (d) There was no obvious abnormality in ADMSCs without osteogenic differentiation (×40).

ADMSCs were placed in an anoxic incubator for 24 h culturing. Then they were observed under a microscope, and it was found that cells still presented “vortex”-shaped growth with morphologies of long fusiform shape retained. A small number of dead cells were floating on the surface of the complete culture solution, and there was no obvious difference in morphologies from cells in the ordinary culture group (as shown in Figure 4).

RADA16-I, RGD, KLT, and the self-assembly polypeptide solution were all colorless transparent liquids, and good self-assembly of the functional polypeptide solution

Figure 3: (a) After ADMSCs differentiate into adipose, the cell morphology is no longer fusiform (×40), and cell morphology changes. (b) After adipogenic differentiation of ADMSCs, the cells changed from spindle to triangle or irregular shape (×200). (c) After adipogenic differentiation of ADMSCs, oil red O staining showed intracellular red lipid droplets (×40). (d) There was no significant change without adipogenic differentiation cells (×40).
could be observed under an atomic force microscope. As shown in Figure 5, RADA16-I, RGD, and KLT were all nanofiber shapes as observed under an atomic force microscope, with fiber diameters of 17.34 ± 1.82, 15.50 ± 1.41, and 13.77 ± 1.18 nm, respectively. The functional self-assembly polypeptide was also of nanofiber shape and the fiber diameter was 20.26 ± 1.25 nm. By the statistical analysis, as in Figure 6, the diameter of the self-assembly polypeptide increased when compared with those of the original three types of polypeptide fibers (P < 0.05).

The ELISA method was used to determine VEGF and HGF concentrations in the cell supernatant. The HGF concentrations in the common culture group, hypoxic culture group, and three-dimensional culture group were 47.31 ± 6.75, 247.86 ± 17.59, and 297.25 ± 17.95 pg/mL, respectively; their VEGF concentrations were 218.30 ± 3.03, 267.13 ± 4.27, and 289.14 ± 3.11 pg/mL, respectively. Thus, it could be seen that VEGF and HGF concentrations in the cell supernatant in both the hypoxic culture condition and three-dimensional culture condition obviously increased when compared with the common culture group (P < 0.05). The details can be seen in Figure 7.

When cells were under the hypoxic culture environment, expression of the intracellular heme oxygenase (HO-1) would obviously increase. According to western blot results analysis, it could be seen that the HO-1 content expressed in cells in both the hypoxic culture group and three-dimensional culture group obviously increased when compared with the common culture condition group. The protein content expressed in the three-dimensional culture group was the highest (P < 0.05) (as shown in Figure 8).
For the treatment of ischemic diseases, including ischemic heart disease and ischemic limb disease, stem cell treatment is a therapeutic method with prospects at present [6,7]. However, after treatment by inoculating stem cells, stem cells proliferate in the damaged part with a low growth rate, so the therapeutic effect cannot be achieved, which is the main disadvantage of restricting stem cell treatment [8,9]. Moreover, the existing studies have found that tumor risk exists after stem cell treatment [10,11]. One of the solutions to overcome the above disadvantage of stem cell treatment is to use the conditional culture medium for stem cell culturing to treat ischemic diseases. Stem cells can express, synthesize, and secrete cytokines and growth factors and regulate multiple types of bioactive factors such as polypeptides [12–15], especially ADMSCs, which can secrete proangiogenic factors and anti-apoptosis factors, including VEGF, HGF, bFGF, and TGF-b. Anoxia is an important factor influencing the secretion of bioactive factors by stem cells [16,17]. The concentration of bioactive factors secreted by stem cells after ordinary culture conditions is low, so it is difficult to exert a therapeutic effect. Recent studies have found that the VEGF concentration in the supernatant under ordinary culture conditions is 217 ± 97 pg/mL [18], while the concentration of VEGF with therapeutic significance is approximately 5,000 pg/mL [19,20]. Therefore, in vitro culturing of MSCs and enhancement in the ability of stem cells to secrete bioactive factors constitute the key to solving stem cell treatment problems. The three-dimensional culturing of ADMSCs by the “microsphere method” and by the use of supernatant obtained from culturing with the three-dimensional conditional culture medium can significantly ameliorate acute ischemic kidney diseases [21]. By three-dimensional culturing of ADMSCs using the “microsphere method,” bioactive factors secreted by stem cells will increase, which is closely related to the anoxic environment where the cells are located [22]. Moreover, three-dimensional culture conditions are similar to the in vivo environment within cells and can be better influenced by the physical environment and biological environment. Therefore, a functional self-assembling nano-polypeptide hydrogel was used in this experiment as a biological framework for the three-dimensional stem cell culture.

According to the previous literature reports, the self-assembling polypeptide RADA16-I can spontaneously form nanofibers with diameters of 10 nm, pore diameters of 5–200 nm, and abundant moisture [23–25]. The formed polypeptide nanofibers are extremely similar to the extracellular matrix and can act as a biological framework for the three-dimensional cell culture. The polypeptide RGD is a key integrin for cell adhesion and can facilitate cell
adhesion [26,27]. The polypeptide KLT is a stimulatory factor of VEGF [28]. In this study, the above three types of polypeptide solutions were sufficiently blended in a volume ratio of 2:1:1, and then the colorless and transparent liquid was obtained. By observing the self-assembling polypeptide solution obtained after blending under an atomic force microscope, the solution still consisted of nanofiber structures. Compared with RADA16-1, the fiber diameter obtained through self-assembly was larger (thick). Thus, it can be seen that the self-assembling nano-polypeptide obtained by blending three types of polypeptides in a volume ratio of 2:1:1 could be very well assembled into a nanofiber structure with abundant moisture and could be used as a biological framework for the three-dimensional cell culture.

In this experiment, the in situ three-dimensional culture of ADMSCs was carried out using a functional self-assembling nano-polypeptide hydrogel, and the ELISA method was used to determine the VEGF and HGF concentrations in the supernatant of the culture medium under three-dimensional culture conditions and ordinary culture conditions. It was found that ADMSCs secreted pro-angiogenic factors more significantly after three-dimensional culture than under ordinary culture conditions. When cells are beyond the oxygen diffusion distance (generally 150–250 μm), they will be in an anoxic state [29]. Therefore, we believe that when a functional self-assembling polypeptide hydrogel is used for the three-dimensional culture, the anoxic state of cells is the influencing factor for increasing the secretion of pro-angiogenic factors. The results for the anoxic group in this study showed that under anoxic conditions, even an ordinary two-dimensional culture would result in an increase in the expression of pro-angiogenic factors by ADMSCs; however, the magnitude of this increase was obviously lower than that under three-dimensional culture conditions. Extensively existing in vivo microsomal enzyme systems, HO, including three types of isozymes, HO-1, HO-2, and HO-3, participates in multiple philological and pathological processes in vivo. HO-1 can be activated by multiple oxidative stress factors in vivo and has important in vivo effects, such as antioxidation, anti-inflammatory reactions, and immune adjustment [30]. We found that HO-1 expression levels in cells in the three-dimensional culture group and the anoxic group were obviously higher than those in the ordinary culture group and so it is believed that the protein kinase (Akt) signal pathway is activated under three-dimensional culture conditions [16]. The expression of anoxic genes was upregulated inside ADMSCs, and as a result, the number of pro-angiogenic factors secreted by ADMSCs increased.

MSCs were obtained through isolated culture from fat tissues, and ADMSCs were validated by combining adherence screening and multidirectional differentiation potential. The mediated three-dimensional culture was carried out for ADMSCs with a functional self-assembling nano-polypeptide hydrogel, and it was found that the level of pro-angiogenic factors secreted by ADMSCs increased under three-dimensional culture conditions. Moreover, anoxia was validated as one of the important factors in increasing secretion. However, a further in-depth study is needed regarding whether the expression of anoxic genes in ADMSCs is upregulated by activating the Akt signal pathway during mediated three-dimensional ADMSC culture conditions using a functional self-assembling nano-polypeptide hydrogel.

5 Conclusion

In this experiment, MSCs were isolated and cultured from adipose tissue, and ADMSCs were confirmed by adherent screening and multidirectional differentiation potential. Three-dimensional culture of ADMSCs mediated by functionalized self-assembled nano-polypeptide hydrogel showed that ADMSCs secreted increased growth of vascular growth factors such as HGF and VEGF under three-dimensional culture conditions and confirmed that hypoxia was one of the important factors for its secretion. However, it is still necessary to further study the function of functional peptide nanometer hydrogels on ADMSC-mediated three-dimensional culture in order to upregulate the expression of Akt in ADMSCs.

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Author contributions: Ling Jianmin performed the experiments, analyzed, and interpreted the data, and wrote the manuscript; Tian Ailing and Yi Xin performed the analysis and interpretation of the data; Sun Nianfeng designed the study and revised the manuscript. All authors read and approved the final manuscript.

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Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical approval: The procedures and participation/tissues of humans in this study were carried out in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory. All experimental protocols were approved by the Ethics Committee of Shandong Academy of Medical Sciences. All participants provided a statement of written informed consent.

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