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Original Article

Effects of Different Cell Types on Exosome Secretion in 2D Cell Cultures

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Abstract: Exosomes are attracting significant attention as their potential therapeutic agents and have the ability to deliver bioactive molecules in a targeted manner. They have roles in regenerative medicine, neurological disorders, cardiovascular diseases, and immunomodulation. However, the biological activities of exosomes depend on their cargos and are affected by the secreting cells. This study aims to investigate the number and size distribution of exosomes released by Human Exfoliated Deciduous Teeth Stem Cells (SHEDs), fibroblasts, and Umbilical Cord-Derived Mesenchymal Stem Cells (UCMSCs). Additionally, we evaluated the expression of several growth factors in exosomes. The results showed that three different cell types released different numbers of exosomes, with the greatest number belonging to UCMSCs. Additionally, exosome particles from all three samples concentrated at peak 130 nm - 180 nm, and more than 90% of the detected particles ranged from 30 nm to 350 nm. Furthermore, expression levels of growth factors, including Fibroblast Growth Factor-2 (FGF-2), Hepatocyte Growth Factor (HGF), Platelet-Derived Growth Factor-BB (PDGF-BB), and Vascular Endothelial Growth Factor-A (VEGF-A) are different. Growth factors seemed to be more enriched in exosomes derived from UCMSCs and fibroblasts than SHEDs. Data from this study indicate that the characteristics and further application of exosomes were affected by the secreting cell types.

Keywords: Exosomes, size distribution, growth factors, fibroblasts, mesenchymal stem cells.

1. Introduction

Exosomes are nano-sized, membrane-enclosed extracellular vesicles released by many types of cells. They are typically 30 nm to 250 nm in diameter and play a crucial role in cell-to-cell communication [1-3]. Biogenesis of exosomes begins with the endocytosis of endocytic vesicles, which then form multivesicular bodies (MVBs). When these MVBs fuse with the plasma membrane, they release the exosomes into the extracellular milieu [4]. Exosomes carry various bioactive molecules; they can influence the behavior of recipient cells. Thus, they are involved in

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various physiological and pathological processes, including immune responses, tumor progression, and neurodegenerative diseases [2]. It is noted that the exosomal content of exosomes reflects the state of secreting cells, enabling their potential use as biomarkers for disease diagnosis and as vehicles for drug delivery.

Exosomes from different cell types can differ in several key aspects, reflecting the distinct roles and states of the originating cells [4]. These include the composition, such as bioactive molecules (proteins, RNAs, and lipids), surface markers, and biological functions. Because of these differences, analyzing the exosome profiles of various cell types can provide valuable insights into cellular functions and disease mechanisms. This has led to significant interest in using extracellular vesicles as diagnostic tools and therapeutic agents.

Exosomes from fibroblasts and mesenchymal stem cells have been investigated for their roles in regenerative medicine and disease treatment [5, 6]. These findings showed that exosomes derived from UCMSCs, adipose-originated MSCs, and bone marrow-originated MSCs expressed their great effects on wound healing and osteoarthritis through facilitating cell proliferation, migration, and extracellular matrix (ECM) gene expression [3, 7]. Additionally, exosomes derived from human dermal fibroblasts could protect skin from photo-aging [8]. A summary showed that exosomes from cancer-associated fibroblasts expressed their roles in regulating the activity of signaling pathways, which promote tumor progression and immune-related inflammation, and participate in signal crosstalk among tumor and immune cells [9]. However, there needs to be a more direct comparison of exosomes originating from different cells, including their size, distribution, and components. Thus, this study investigates the effects of other cells, such as SHEDs, fibroblasts, and UCMSCs, on the exosome secretion, including number, size distribution, and expression of growth factors. The purpose of comparing exosomes secreted by fibroblasts**,** UCMSCs, and SHEDs in terms of size and growth factor content is to identify their distinct regenerative capabilities and to better understand how these exosomes could be tailored for specific therapeutic applications. By analyzing factors such as FGF-2**,** HGF**,** PDGF-BB, and VEGF-A, researchers can select the most appropriate exosome sources for targeted treatments, ultimately optimizing exosome-based therapies for tissue regeneration, wound healing, and other applications in regenerative medicine.

2. Experiementation

2.1. Human Fibroblast Isolation and Expansion

Human fibroblasts (hFBs) were isolated from dermal skin and cultured in conventional media (DMEM/F12 supplemented with 10% FBS and 1% Pen/Strep). Firstly, the skin was sectioned into small pieces (approximately 0.5 cm^2), and the epidermis was removed. Then, the dermis was sectioned into small and thin pieces and incubated at 37 ^oC overnight with DMEM/F12 (Gibco, USA) + 10% FBS (Gibco, USA) + 200U collagenases (Gibco, USA). Then, dermis pieces were collected using centrifugation at 400 × *g*/10 min and seeded into a T25 flask (Nunc, Thermo Scientific, Massachusetts, USA) for cell expansion at 37 °C and 5% CO_2. When cells reached 80% confluency, cells were split to the next passage.

2.2. Mesenchymal Stem Cell Expansion

The primary UCMSCs were supplied by the Extracellular Membrane Vesicle (EV) group (Vinmec Hi-Tech Center). Human Exfoliated Deciduous Teeth Stem Cells (SHED) were supplied by the Cell Manufacturing Department (Vinmec Hi-Tech Center). Cells were expanded to passage five in StemMACS MSC expansion media kit XF (Miltenyi, Germany) in T75 or T225 cell culture flasks (Nunc, Thermo Scientific, Massachusetts, USA) surface-coated with CTS™ CELLstart™ substrate (Gibco, Massachusetts, USA) at the condition 5% CO_2 and 37 ^oC. Cells were split when they reached

80% confluency using CTS TrypLE Select Enzyme (Thermo Fisher Scientific, USA).

2.3. Conditioned Media Preparation

Fibroblasts at passage 3 (P3), UCMSCs, and SHEDs at P5 were seeded at a density of 5000 cells/cm^2 and were cultured to reach 80% - 90% confluency without media replacement during the cultures, approximately 4 to 5 days at 5% CO_2 and 37 ^oC. Then, the supernatant, considered as conditioned media, was collected into the sterilized falcon tubes and stored at 4 ^oC for up to 48 hours for exosome isolation.

2.4. Exosome Isolation

Exosome isolation was performed as previously described in our study [3]. Supernatant or conditioned media collected from cell cultures were centrifuged at 300 × *g*/10 min/4 °C to remove cell debris, and then apoptotic bodies and microvesicles were removed via sequential centrifugation at 2,000 × *g*/20 min/4 °C and 16,500 × *g*/30 min/4 °C, respectively. The remaining supernatant was collected and centrifuged at 100,000 × *g*/90 min/4 °C to isolate exosomes (Optima XPN-100 Ultracentrifuge, Beckman Coulter, California, USA). All exosomes were resuspended in PBS and stored at -80 °C for further use.

2.5. Nanoparticle Tracking Analysis

Exosomes were thawed on ice and thoroughly mixed. They were then incubated with Cell Mask Green (CMG) in a dark room, with a ratio of 9 µL exosomes to 1 µL CMG, for 1 hour. The stained exosomes were subsequently diluted with PBS to achieve a concentration of 10^5 - 10^9 particles/mL. The next step involved injecting 1 mL of the exosome suspension into the sample chamber and setting up the running at Fluorescent Mode with a laser to detect the fluorescence signal at 488 nm and Scattering Mode. Nanoparticles were captured, and their size was measured based on Brownian motion. Parameters were set up for exosome analysis based on 100 nm standard particles.

2.6. Statistical Analysis

Statistical analysis was performed using GraphPad Prism (v.8.4.3; GraphPad Software, San Diego, California). The data were collected from three biological repeats and presented as the Mean ± SD and analyzed by one-way analysis of variance (ANOVA), followed by a Tukey multiple comparison test. $p < 0.05$ was considered a statistically significant difference.

3. Results and Discusion

3.1. Efficacy of Different Cell Types to Secret Exosomes into Culture Media

We used nanoparticle tracking analysis (NTA) to evaluate the efficacy of different cell types in secreting exosomes into conditioned media (Figure 1A1, 1A2, and 1A3). Exosomes were analysed in scatter mode to determine the total number of particles detected. Data showed that three cell types affected the particle release, with UCMSCs releasing the greatest number. Fibroblasts secreted exosomes more than SHEDs did, but the difference is not significant.

Additionally, we analyzed particles stained with CMG. CMG is a dye that binds to the lipid membrane, and the exosomes that were labeled with CMG were measured in fluorescent mode for CMG-positive lipid membrane particles. Data showed that fewer particles were positive for CMG (Figure 1C) than were detected using a size filter only (Figure 1B). Additionally, the fluorescent signals from particles derived from SHEDs were lower than those from fibroblasts and UCMSCs, but the smaller number of CMG-positive particles did not reach a statistical difference. These data indicate that among all particles detected, some may be non-extracellular particles contaminating the exosome fraction.

Extracellular vesicles, including exosomes, are emerging as potential therapeutics and diagnostics due to their cargos that reflect the origins and pathological state of secreting cells. In this study, we investigated the number of exosomes released by three different cell types,

including SHEDs, fibroblasts, and UCMSCs, in 2D cell cultures. Interestingly, the number of exosomes released by UCMSCs is highest compared to SHEDs and fibroblasts, but a similar number of these particles are positive with CMG; this may be due to the contamination of particles with non-lipid membrane, for example, protein aggregates, into the exosome fraction. Previously, studies have analyzed the number of exosomes released by different cells under specific conditions. The number of exosomes secreted by C2 cell lines (a canine mast cell tumor cell line) was 2 - 3-fold higher than that of primary canine fibroblasts after 48 hours and 72 hours of culture [10]. In hypoxic conditions, HT29 and HCT116 cells released fewer exosomes [11], whereas breast cancer cell lines (MCF7, SKBR3, and MDA-MB 231) released more exosomes [12]. Unfortunately, there was no direct comparison of exosome numbers associated with primary mesenchymal stem cells and fibroblasts.

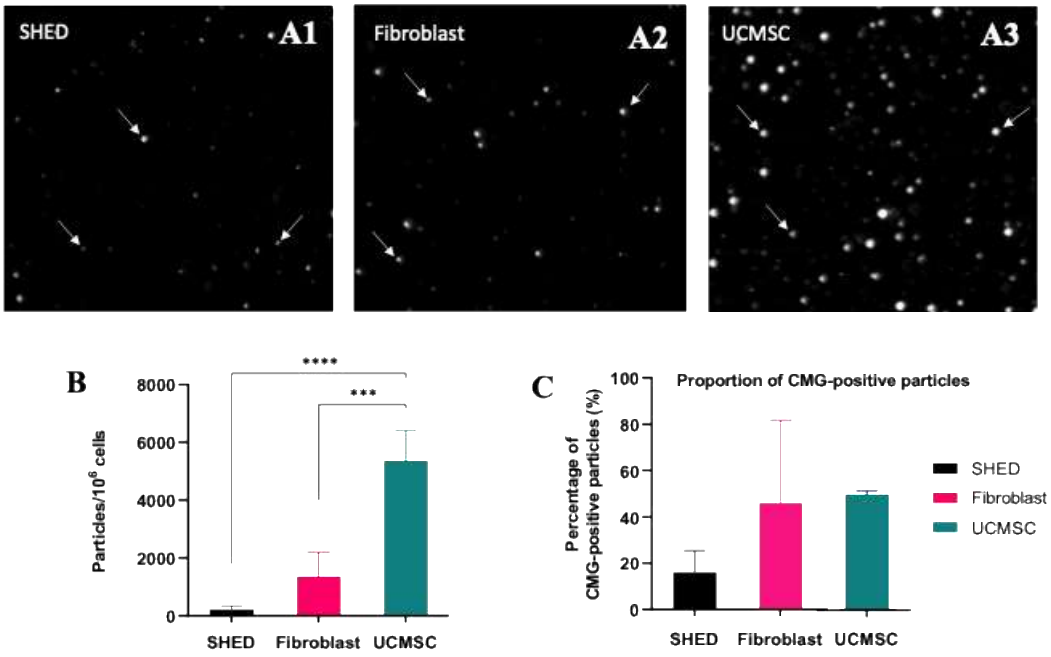

Figure 1. Observation of exosome concentration under NTA (n = 3).
A1, A2, A3) Images of EV observed under NTA, arrows indicate exosome particles captured by NTA.
B) Number of particles detected per 10^6 cells and C) Percentage of particles positive with CMG.

3.2. Exosome Size and Distribution

After analyzing the capacity of cells to release exosomes, we examined the size distribution of exosomes derived from different cell sources. Data showed that more than 90% of particles in the exosome size range from 30 nm - 350 nm were detected in all three samples of SHEDs, fibroblasts, and UCMSCs (Figure 2). Additionally, particles concentrated at the peak around 150 nm - 180 nm (Figure 2A). There were proportions (less than 10%) of larger particles (>350 nm) contaminated in the exosome fraction, and particles smaller than 30 nm were less than 0.3% in all samples (Figure 2B). These data are consistent with the above statement that particles may be contaminated in exosome fractions.

Regarding the size distribution of exosomes, it is noted that the particle size depends on the detection technology and cell type [13]. Typically, researchers use an electron

microscope, surface plasmon resonance, flow cytometry, tunable resistive pulse sensing, and NTA to quantify and determine exosome size. However, there is currently no consensus on either an optimal approach or how to properly compare results using different approaches [13]. This study used NTA technology to detect the size of exosomes that are from 30 nm to 350 nm, which accounted for more than 90% of the total particles detected, with a peak around 150 nm - 180 nm (Figure 2). This is similar to another study in which exosomes measured by NTA technology expressed their size from 33 nm to 380 nm [14]. Due to the isolated exosomes using differential centrifugation, this could not eliminate all other non-EV particles contaminated in the exosome fractions; this may lead to larger or smaller particles that are out of the exosome size range.

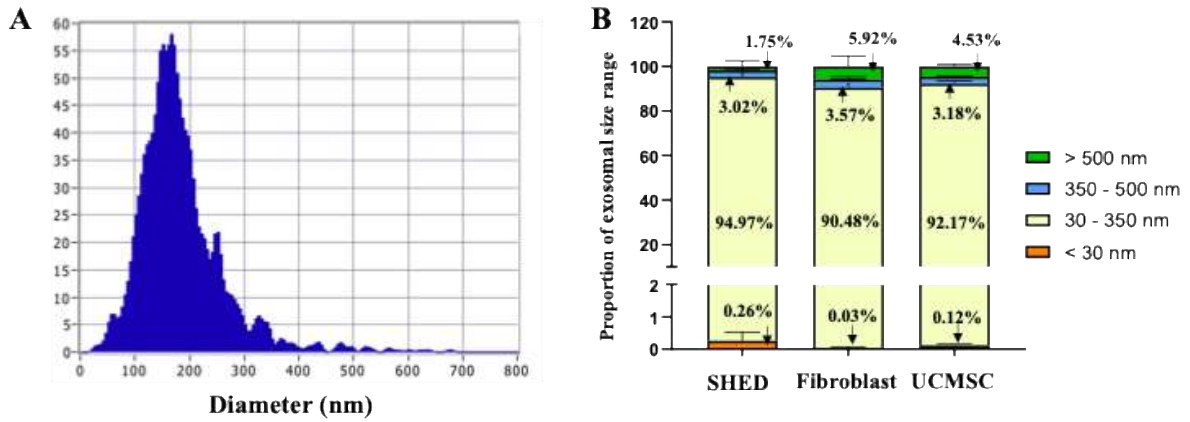

Figure 2. Size distribution of exosomes under NTA analysis (n = 3).
A) Graphic analysis of exosome size distribution,
a representative of exosomes derived from fibroblasts.
B) Proportion of size range in a total of EX suspension,
where the majority of particles are in the range of 30 nm - 350 nm.

3.3. Expression of Growth Factors in Exosomes Derived from Different Cell Types

We examined the growth factor concentration in exosomes to understand how cell type affects growth factor secretion. Results showed that all four growth factors, including FGF-2, HGF, PDGF-BB, and VEGF-A, were detected in exosomes. However, the expression of these factors is different (Figure 3). The level of PDGF-BB was detected much more in exosomes derived from UCMSCs compared to exosomes derived from SHEDs and fibroblasts. Additionally, FGF-2 and HGF were also detected with higher levels in UCMSCs, and VEGF-A was detected with a higher level in fibroblasts, but these differences were not significant. Thus, these data indicate that the level of growth factors may be related to the cell type.

These growth factors, including FGF-2, HGF, PDGF-BB, and VEGF-A, have been reported in exosomes derived from MSCs and fibroblasts [3, 15, 16]. To our knowledge, there was no report on these growth factors presented in exosomes derived from SHEDs, and our study is the first one. Additionally, growth factors of FGF-2, HGF, PDGF-BB, and VEGF-A that have important roles in regenerative medicine, for example, wound healing, seem to be expressed at higher levels in UCMSCs and fibroblasts. Particularly, the much higher expression of PDGF-BB and VEGF-A in exosomes secreted by UCMSCs and fibroblasts, respectively, may be the exosomes' cargo based

on cell-specific origins and functions in wound healing and ECM remodeling. This makes UCMSC- and fibroblast-derived exosomes more specialized for tissue repair processes that require PDGF-BB and VEGF-A signalings. This may reflect the different roles of SHED-derived exosomes compared to UCMSC- and fibroblast-derived exosomes. However, further investigations are required on these components and other exosomal contents and their relevance to secreting cell types, to guide the application of different cell-derived exosomes for different disease treatments.

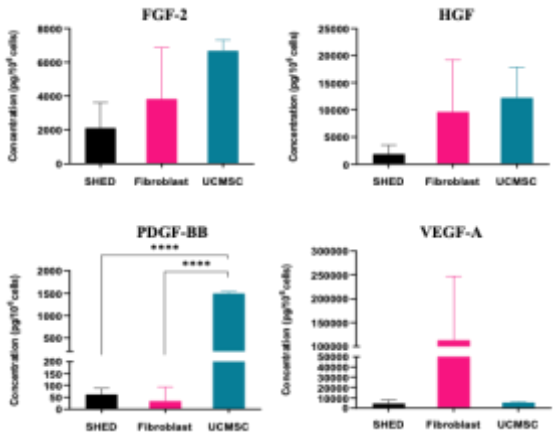

Figure 3. Growth factor expression in exosomes derived from different cell sources. PDGF-BB enriched more in exosomes derived from UCMSCs than those derived from fibroblasts and SHEDs (n = 3; **** indicates $p<0.0001$.

4. Conclusion

Particles were detected in exosome fractions isolated from 2D cultures of primary SHEDs, human dermal fibroblasts, and UCMSCs, with the size of exosomes and positive with lipid binding dye (CMG). More particles were detected in the exosome fraction from UCMSCs, but the particles positive with CMG were similar among all exosomes derived from the three cell sources. Additionally, the growth factor of PDGF-BB was expressed more in UCMSCs, and it seems other growth factors (FGF-2 and HGF) were enriched more in exosomes derived from UCMSCs and fibroblasts than SHEDs. This requires more investigation into the relevance of these growth factors to the tissue sources of these cells.

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