Human adipose mesenchymal stem cell-derived exosomal-miRNAs are critical factors for inducing anti-proliferation signalling to A2780 and SKOV-3 ovarian cancer cells

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An enigmatic question exists concerning the pro- or anti-cancer status of mesenchymal stem cells (MSCs). Despite growing interest, this question remains unanswered, and the debate became intensified with new evidences backing each side. Here, we showed that human adipose MSC (hAMSC)-derived conditioned medium (CM) exhibited inhibitory effects on A2780 human ovarian cancer cells by blocking the cell cycle, and activating mitochondria-mediated apoptosis signalling. Explicitly, we demonstrated that exosomes, an important biological component of hAMSC-CM, could restrain proliferation, wound-repair and colony formation ability of A2780 and SKOV-3 cancer cells. Furthermore, hAMSC-CM-derived exosomes induced apoptosis signalling by upregulating different pro-apoptotic signalling molecules, such as BAX, CASP9, and CASP3, as well as downregulating the anti-apoptotic protein BCL2. More specifically, cancer cells exhibited reduced viability following fresh or protease-digested exosome treatment; however, treatment with RNase-digested exosomes could not inhibit the proliferation of cancer cells. Additionally, sequencing of exosomal RNAs revealed a rich population of microRNAs (miRNAs), which exhibit anti-cancer activities by targeting different molecules associated with cancer survival. Our findings indicated that exosomal miRNAs are important players involved in the inhibitory influence of hAMSC-CM towards ovarian cancer cells. Therefore, we believe that these comprehensive results will provide advances concerning ovarian cancer research and treatment.

Different organs including ovaries are surrounded and supported by adipose fat-pad, which provide physical, as well as mechanical supports and play important roles during organogenesis, morphogenesis, disease progression of respective organs. Being an important composite of adipose-stromal cells, adipose mesenchymal stem cells possibly have regulatory part in different cancers, such as ovarian cancer. However, relationships between mesenchymal stem cells (MSCs) and cancer cells are a mystery, owing to insufficient evidence concerning both the stimulatory and inhibitory roles of MSCs on cancer cells. While there is debate about the customary roles of MSCs, their involvement in cancer biology is undoubtedly clear. MSCs potentially support tumour development through immune suppression, epithelial-to-mesenchymal transition, angiogenesis, and serving as cancer stromal cells. In contrast, MSCs also suppress cancer by downregulating cancer survival-signalling pathways involving WNT/β-catenin and/or AKT. There is a need to investigate the mechanisms underlying the contradictory roles associated with MSCs in cancer biology. Cytokines and soluble factors secreted by MSCs have been thoroughly scrutinized, with most reports concluding that MSC-secreted cytokines and soluble factors exhibit stimulatory effects related to cancer progression.

Exosomes are types of membrane-bound micro-vesicles 30 nm to 200 nm in diameter, found in bio-fluids and contain many important components, including RNA, proteins, DNA, and lipids, and serve as efficient vehicles for cancer-stromal communication. Exosomes are secreted by all cells and, despite their ability to be incorporated...
cells were seeded per 10-cm culture dish and incubated overnight at 37°C and 5% CO₂ for proper attachment. Media was replaced with new media, and cells were cultured for an additional 48 h, after which the resulting medium was considered hAMSC-CM. The pH stability was confirmed using both an electric pH meter and pH paper. A total of 11 CM was collected, filtered, mixed, and aliquoted in 15-mL and 50-mL tubes for storage at −20°C.

CM dosage determination and cell-viability assay. To detect the effect of hAMSC-CM on A2780 cell proliferation, a cell-viability assay was performed using 0% to 30% of CM supplementation to the fresh medium. A2780 cells were seeded (1 x 10⁴ cells/well) onto 96-well, flat-bottomed culture plates and incubated for 24 h at 37°C in a 5% CO₂ incubator. The medium was replaced with control (fresh) or treatment (0–30% CM) medium, and cell viability was detected using a cell counting kit-8 (CCK-8; Duinglo Laboratories, Tokyo, Japan). Absorbance was measured at 450 nm in a microtiter plate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA). Based on obtained results, 25% CM supplementation to fresh medium was used for all experiments.

ROS detection. ROS detection was quantified using 2',7'-dichlorodihydrofluorescein diacetate (H2-DCFDA; Sigma-Aldrich). Cells were incubated with 10 μM H2-DCFDA at 37°C in a humidified CO₂ incubator for 30 min. Cells were then washed and re-suspended in phosphate-buffered saline (PBS), followed by detection of fluorescence emission and excitation at 515 nm and 488 nm, respectively, using a Gemini EM (SpectraMAX; Molecular Devices, Sunnyvale, CA, USA).

MMP assessment. MMP was detected by the cationic fluorescent indicator JC-1 (Molecular Probes, Eugene, OR, USA). Cells were incubated with 10 μM JC-1 at 37°C for 15 min, washed with and re-suspended in PBS, and the fluorescence intensity was measured. MMP was expressed as the ratio of the fluorescence intensity of the JC1 monomers to JC1 aggregates. JC1 aggregates indicate red fluorescence for intact mitochondria, with emission at 583 nm, while JC1 monomers demonstrate green fluorescence in cytoplasm, with emission at 525 nm and excitation at 488 nm.

Cell cycle. A2780 cells were seeded onto 6-well plates (30,000 cells/cm²) and allowed to grow overnight, after which the medium was replaced with control (fresh) or treatment (25% CM) medium every 24 h. Cells were harvested at 48 h and 72 h for cell cycle analysis, washed with PBS, fixed with 70% ethanol, and stored at −20°C overnight. Fixed cells were washed again with PBS, treated with RNaseA solution, and stained with propidium iodide. Cell-cycle analysis was performed using BD FACSCalibur (BD Biosciences, San Jose, CA, USA). The raw data was analysed using ModFit software trial version (http://www.vsh.com/products/mft/index.asp).

TMR red assay. A2780 cells were seeded onto 6-well plates (30,000 cells/cm²) and allowed to grow overnight. Medium was replaced with control (fresh) or treatment (25% CM) medium, and the medium was replaced every 24 h. At 72 h, cells were harvested and apoptosis rate was detected using an in situ cell-death detection kit (TMR red; Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer instructions.

Immunoblotting. Radio-immunoprecipitation buffer was used to lye the cells, and protein concentrations were measured with the BCA protein assay kit (Thermo Scientific). Proteins were resolved using 12% SDS-PAGE and transferred electrophoretically to polyvinylidene fluoride membranes. Membranes were incubated with 5% skim milk for 2 h at room temperature (RT), then overnight at 4°C with primary antibodies, and 1 h with horse-radish peroxidase-conjugated secondary antibody at RT. Immuno-reactivity was detected by enhanced chemiluminescence reagents.

Exosome isolation and characterization. Exosomes were isolated according to manufacturer instructions (ExoQuick-TC kit). Briefly, ExoQuick-TC reagent was added to hAMSC-CM at a 1:5 ratio and mixed by inverting the tubes several times. The mixture incubated overnight at 4°C, centrifuged at 1500 g for 30 min at RT, and exosomes were recovered. For TEM, an exosome-TEM grid was prepared according to manufacturer instructions (Exosome-TEM easy kit; 101Bio, Palo Alto, CA, USA), and exosome particles were visualized using an Hitachi H7600 electron microscope (Hitachi, Tokyo, Japan) at 100 kV accelerating voltage.

Cell-viability assay. Exosomes derived from each mL of CM were designated as 1 U exosomes. For viability assay, cells were treated with exosomes at one U/mL. When using protease- or RNase-digested exosomes, the control medium was supplemented with equal amounts of protease or RNase solution to avoid experimental error due to the presence of protease or RNase. Protease-digested exosomes were prepared by digesting 5 U exosomes with 100 μL trypsin (0.25%) at 37°C for 30 min. RNase-digested exosomes were obtained by digesting 5 U exosomes with 100 μL RNaseA solution (100 μg/mL) at 37°C for 30 min.

Wound-repair assay. For detecting wound-repair capacity, cells were seeded (30,000 cells/cm²) on 12-well plates and incubated overnight at 37°C at 5% CO₂. Wounds were made using 10-μl pipette tips, cells were washed with PBS, and fed with either control (fresh) or different treatment combinations (25% CM, 1 U/mL exosomes, or 1 U/mL exosomes plus 25% CM).

Colony formation assay. For colony formation assays, 1000 cells were seeded onto 60-cm cell-culture dishes and incubated overnight at 37°C at 5% CO₂. Medium was replaced with either control (fresh) or different treatment combinations (25% CM, 1 U/mL exosomes, or 1 U/mL exosomes plus 25% CM), and the cells were cultured for 2 weeks. Dishes containing colonies were stained with 0.05% (w/v) crystal-violet solution, including