Evaluation of Pro-regenerative Activities of Human Placenta Derived Exosomes

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ASEMV 2019

ABSTRACT

Placenta is a highly specialized and essential organ for mammalian reproduction. Placenta not only consists of tissue specific cells including cytotrophoblast, syncytiotrophoblast, endothelial cells and epithelial cells, it also has abundant hematopoietic and multipotent stromal stem cells. Placenta derived exosomes are known to play key roles to maintain maternal-fetal tolerance and fetus development during pregnancy. It is perceivable that these exosomes are produced by all cell types in a placenta. Therefore, exosomes isolated from placenta may contain a broader spectrum of biological functions and therapeutic potentials than those from a specific cell type. Here we report the development of methods to isolate placenta derived exosomes (pExo) and characterization of pExo. Tissues processed from human postpartum placenta were cultured at 37ºC with several media collection every 8 to 16 hours for upto 4 days. pExo were purified from the culture media by differential ultracentrifugation and was shown to yield about 300 mg of exosome per placenta as determined by Bicinchoninic Acid protein assay. The average particle size was about 120 nm as determined with nanoparticle tracking analysis. It was shown that pExo expresses characteristic exosome markers including CD9, CD63 and CD81 using flow cytometry. Proteomic analysis showed pExo contains approxiamtely 1,200 different proteins that are involved in different biological pathways. ELISA and MILLIPLEX-MAP assays were utilized to examine over 40 different cytokines, chemokines and growth factors. Among these IL-8, GRO, RANTES, MCP-1, G-CSF, PDGF-BB, IL-6 and FGF-2, were shown to be more abundant compared to other factors. In functional evaluation studies, pExo showed chemotactic activity of stimulating migration of HUVECs and human dermal fibroblasts across membrane of transwell. pExo promotion of cell proliferation was also demonstrated for HUVECs, human dermal fibroblasts and renal epithelial cells. In summary, we have established an effective method to obtain pExo at large quantity with unique cellular composition and pro-regenerative activities supporting further development of pExo in potential functional regeneration applications.

MATERIALS & METHODS

Placenta culture: Full-term human placentas were obtained under the full consent of donors from *LifeBank USA*. Placentas tissue were cultured in serum free cell culture medium supplemented with antibiotics. After culturing 8 to 16 hours, supernatant were harvested, and new serum free media replaced when needed. For continued culture, media were changed every 8 to 12 hours for up to 4 days.

Exosome Isolation: Culture supernatants were centrifuged at 3000g for 30 minutes to pellet cell and tissue debris followed by centrifugation at 10,000g for 1 hour to pellet cellular organelles and large cellular vesicles. The 10,000g supernatant was centrifuged at 100,000g for 2 hours and the pellet is then washed twice with PBS and pass through a 0.22um filtration system. The final exosome preparation was resuspended in PBS and stored at -80ºC.

Exosome quantification and characterization: Quantification of exosomes was performed with BCA protein kit (Invitrogen). The size of exosomes was determined using NanoSight Exosome Analysis provided by System BioScience Inc (SBI).

Proteomic Analysis of Exosomes: The protein contents of isolated placenta exosomes were performed using proteomic service provided by SBI. 10 ug of protein from each exosome samples were analyzed with a Nano-LC-MS/MS and analyzed with Mascot DAT and Scaffold software.

Cytokine characterization and quantification: MILLIPLEX-MAP human cytokine/chemokine-PX41 (EMD-Millipore) was used to analyze the pExo samples from different donors. ELISA kits (Sigma; R&D systems) were also used to analyze other growth factor and cytokine.

Cell Migration Assay: 200 uL of 1x10e5/mL human HUVEC expressing GFP in basal media were seeded on an 8um transwell on the top camber of a 24-well plate with 400uL of DMEM basal medium with or without 100ug of placental exosomes. After 4 hours of culture, transwells were are observed under an inverted microscope.

Cell Proliferation Assay: Human primary cells were seeded at 2000-4000 cells/96-well. After overnight culture, media were removed, washed three times with PBS and then replaced with basal medium (BM) supplemented with pExo at different concentrations. The viability and total cell were assessed with WST cell proliferation assay (Sigma). OD540 data normalized to basal medium as control after subtraction of background.

RESULTS

1. Procedures have been established to cultivate human placenta to isolate exosome by sequential centrifugation (Figure 1). Significant amount of exosomes can be generated from one single placenta as determined and estimated by protein quantification assay using a BSA protein standard. The average yield from one placenta was about 300 mg (N=10).

FIGURE1. Isolation of exosomes from cultivated human placenta. *Preparation of a human placenta for culture (left) and exosome pellets after 100,000 g ultracentrifugation (right)*

2. Size distribution of placenta exosomes were determined by Nanoparticle Tracking Analysis (NTA) using a NanoSight instrument performed by SBI. 3 videos were analyzed for each sample. Three placenta exosome isolates were analyzed. The results showed that the particles has a mean/MOD size about 120 nm. This was consistent with the consensus size of exosomes. An example of NTA analysis of size distribution (Figure 2, left) and the visualization of particles are shown (Figure 2, right)

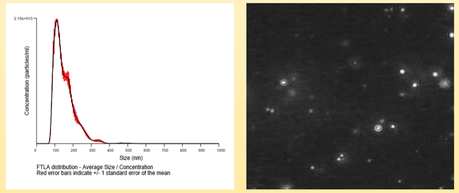

FIGURE2. Size distribution of placental exosomes. *NTA analysis of a representative placenta exosomes isolate (left) and image snap of video of NanoSight analysis (right).*

3. Protein marker of pExo was analyzed with Human MACSPlex Exosome Kit (Miltenyi). 100ug of pExo samples were incubated with the capturing beads provided in the kit at room temperature overnight. The beads-exosome mixture was washed three times followed with anti-CD9, CD63 and CD81 antibodies and then analyzed with BD FACS-Canto (Beckman Dickson). The data showed in Figure 3 is average of 9 different pExo samples

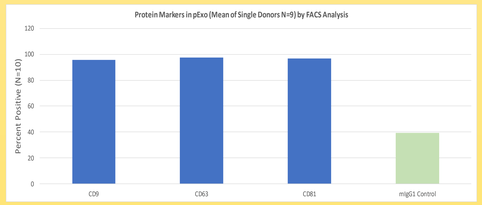

FIGURE3. pExo are positive for CD9, CD63 and CD81 by FACS analysis. *The data is mean percentage of positive events of pExo from 9 different donors. mIgG1 control is the controls beads. Blank (PBS) signals are subtracted from each sample.*

4. Proteomic analysis were performed on three different placental exosome isolations. Preliminary analysis showed that there were about 1100 to 1300 identified proteins in these preparations. Common proteins were identified protein while there some unique proteins in each preparation. Proteins specific to human placenta are present in all three samples. CD9, CD63 and CD81 were also identified in the common proteins. These data confirmed the origin and characterization of the exosome (Figure 4, Left). The functional profiling of the protein cargo of these three placenta exosomes are shown in Figure 4 (Right).

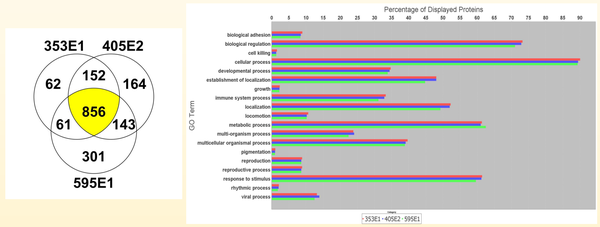

FIGURE 4. Common and unique proteins among three placental exosomes demonstrated by proteomic analysis and key biological pathways these proteins are implicated.

5. Human placenta exosomes demonstrated an activity in stimulating the migration of human HUVEC in a transwell migration assay. Only limited cells migrated to PBS control medium (Figure 6).

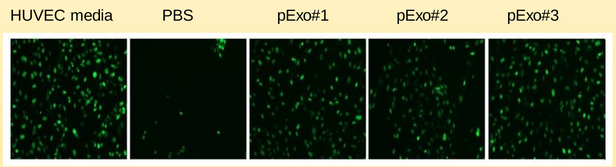

FIGURE 5. Human placental exosomes stimulated migration of human umbilical cord vessel endothelial cells (HUVEC).

6. pExo promoted proliferation of human primary cells. pExo showed activities in promoting the proliferation of human dermal fibroblast (dose dependent) and umbilical blood vessel endothelial cells (HUVEC).

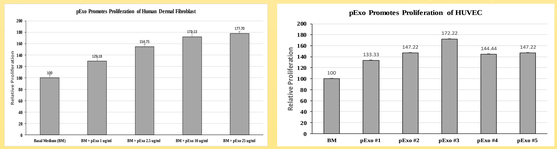

FIGURE 6. pExo promoted human dermal fibroblast proliferation (Left) and HUVECS (Right).

7. Selective cytokine and chemokines in pExo and their functions

Name	Activities	Target Cells
IL-8	Proinflammatory; Angiogenic	Neutrophil; Vascular endothelial cells
GRO	Proinflammatory; Angiogenic	Neutrophil; Vascular endothelial cells
RANTES	Chemotactic	T cells, Eosinophils, NK cells
MCP-1	Chemotactic; Neuorprotetive	Monocyte
G-CSF	Hematopoietic	Hematopoietic cells
PDGF-BB	Chemotactic; Mitogenic; Neuoprotive	Fibroblasts; Smooth Muscle Cells; Glial Cells
IL-6	Inflammation, hematopoiesis, bone formation	Hepatocytes; Monocytes; Lymphocytes
FGF2	Mitogenic; Angiogenic	Cells of mesodermal, neurodermal, enctodermal and endodermal origin

DISUSSIONS & CONCLUSIONS

In recent years, investigators in the stem cell therapy have noticed that the functions of mesenchymal stems cells are mediated by the secreted factors and micro-vesicles. Many publications have demonstrated that stem cell derived exosomes display at least partial functions of the cells in vitro and in vivo. These findings suggest novel exosome based therapeutic opportunities (1-5). .

Human placenta is the organ that plays pivotal role in nurturing the development of fetus from embryonic stage until full term delivery. Both hematopoietic stem cells and non-hematopoietic stem cells have been discovered, characterized and developed into clinical stage products (6, 7, 8) at Celularity Inc (formally Anthrogenesis Inc and Celgene Cellular Therapeutics).

While cell derived exosomes are demonstrated to have the functions of stem cells, it is unlikely that these exosomes harbor the complexity of tissue or organ. It is reasonable to postulate that exosomes isolated from an organ like human placenta could generate an exosome pool with broader functions.

In this study, we demonstrate that exosomes can be isolated and purified from postpartum human placenta by cultivation of the placenta. These placental exosomes are consistent with the characterization of cell derived exosomes in both size and protein markers. Proteomic analysis have shown that placentas exosomes contain proteins that play key roles in many cellular process such as developmental process and immune process. pExo contains cytokines and chemokines that play important roles in promoting angiogenesis, tissue repair and immune-regulation which are consistent with their activities of promoting cell migration and proliferation in vitro.

These dada suggest that human placenta exosomes (pExo) could be developed as next generation of biological therapeutics

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