Supplementary information

Exosomes released by melanocytes modulate fibroblasts to promote keloid formation: a pilot study

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Materials and methods

Tissue specimens

The normal scar tissues and the keloids were obtained from twelve donors (six donors in each group) undergoing routine surgery after written informed consent, with approval from the Clinical Research Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine and in accordance of the tenets of the Declaration of Helsinki.

Supplemental Table 1 Characteristics of scar/keloid samples from twelve donors

Sample	Patient number	Ethnicity	Sex	Age (years)	Body site of scar/keloid	Size of scar/keloid (cm)
Scar	1	Han Chinese	Male	23	Middle chin	0.8×0.8
	2	Han Chinese	Male	27	Right face	6.0×0.5
	3	Han Chinese	Female	37	Left buttock	3.0×3.0
	4	Han Chinese	Female	39	Lower abdomen	8.7×0.8
	5	Han Chinese	Female	64	Left lateral eyelid	1.5×1.0
	6	Han Chinese	Female	21	Right face	4.0×0.5
Keloid	7	Han Chinese	Female	26	Left shoulder-back	3.5×3.0×0.5
	8	Han Chinese	Female	56	Middle chest	10.0×2.5×0.5
	9	Han Chinese	Female	30	Middle neck	5.0×0.5×0.5
	10	Han Chinese	Male	43	Right chin	4.5×4.0×0.5
	11	Han Chinese	Female	54	Low back	6.0×2.0×0.5
	12	Han Chinese	Female	35	Right auricle	4.0×2.0×1.5

Histochemistry

The specimens were fixed in formalin and embedded in paraffin before being cut into 3 μm slices. For H&E staining, after dewaxing and rehydration, the sections were stained in hematoxylin for 8 min and were washed with running tap water for 5 min. Next, the sections were stained in eosin for 30 s. Finally, the sections were dehydrated and mounted using neutral resins.

Immunofluorescence assay

Cells were fixed with 4% paraformaldehyde in PBS for 15 min and then permeabilized with 0.2% Triton X-100 in PBS for 15 min, blocked with 4% BSA in Tris-buffered saline for 0.5 h and incubated with primary antibodies at 4 ℃ overnights. After washing with PBS, the cells were incubated with secondary antibodies for 1 h at room temperature, stained with DAPI for 5 min and mounted for fluorescence microscopy.

Transmission electron microscopy

The exosomes or tissue specimens were fixed in 4% glutaraldehyde overnight at 4 ℃. The exosomes sample (10 μL) was dripped and precipitated on a copper net for 1 min; the floating liquid was absorbed. Similarly, hydrogen peroxide acetate (2%, 10 μL) was dripped and precipitated on the copper net for 1 min. The floating liquid was absorbed, allowed to dry at room temperature for several minutes. Tissue specimens post-fixed in 2% osmic acid, dehydrated, embedded, sectioned at a thickness of 1 μm. Then stained with uranyl acetate and lead citrate and examined under an electron microscope.

Cell culture

Human primary fibroblasts were obtained from keloids by Fibroblast Isolation Kit (Miltenyi Biotec). Human primary melanocytes were obtained from the foreskin tissue of healthy young children by the method of two-step enzymatic digestion. Only melanocytes from the second to tenth passage were used. Melanocytes were seeded in six-well plates at 1.5×10^5 $well^{-1}$, and the medium was replaced with fetal bovine serum free culture medium 3 d before we collected the medium for subsequent experiments. Fibroblasts were cultured in a normal culture medium (control) or an M1–M4 conditioned medium for 48 h. M1–M4 indicate the culture supernatant of melanocytes diluted in a normal medium at different concentrations (M1 1:10, M2 1:4, M3 1:2, M4 3:4).

Exosome isolation

The melanocytes medium was replaced with fetal bovine serum free culture medium 3 d before we collected the medium once for exosome isolation. Exosomes were isolated using differential centrifugation, as follows: The media were centrifuged at 300*g* and 3000*g* for 15 min at 4 ℃, and the precipitates were removed. The supernatant was then centrifuged at 10000*g* for 30 min at 4 ℃, and the precipitates were again removed. Subsequently, the supernatant was filtered by passing through a vacuum-connected 0.22 μm filter. The supernatant was again centrifuged at 120000*g* for 30 min at 4 ℃. The precipitates were collected, resuspended, and then washed in PBS and centrifuged at 120000*g* for 30 min at 4 ℃, and then the precipitates were collected. The exosomes were resuspended in PBS and

stored at −80 ℃ until further use. And the supernatant obtained in the last step of exosome purification was the culture supernatants depleted of melanocyte exosomes.

Co-culture of fibroblasts and melanocytes

CD63-GFP-transduced melanocytes (green) in mono- or co-culture (ratio 1:1, fibroblasts incubated with the same number of melanocytes) with fibroblasts were stained for tubulin (red) and were analyzed using immunofluorescence assay. The distance of CD63-positive compartments from the center of the corresponding nucleus was quantified in melanocytes in mono- or co-culture with fibroblasts.

Western blot analysis

Cells or exosomes were lysed on ice in a lysis buffer with a protease inhibitor cocktail. The cell lysates or exosomes were incubated in a loading buffer and then boiled for 10 min. Total protein was extracted, and the protein concentration was assayed using a BCA kit (Thermo Fisher Scientific, Waltham, MA, USA). Following polyacrylamide gel electrophoresis for 35 min at 80 V and for 45 min at 120 V, protein was transferred onto nitrocellulose membranes (Millipore, USA). The membranes were blocked in PBS-Tween 20 (PBST) (PBS buffer containing 0.1% Tween-20) with 5% non-fat milk for 1 h at room temperature. Subsequently, the membrane was incubated with the indicated primary antibody overnight at 4 ℃. The membrane was washed three times with PBST for 10 min for each round and then incubated in HRP-conjugated secondary antibody for 1 h. Finally, blots were developed by using the ECL and then scanned under an optical luminescence instrument. Three parallel experiments were performed.

Real-time PCR

The total cellular RNA was extracted using a Total RNA Kit II and was reverse-transcribed into cDNA using a reverse transcription kit. The PCR system was prepared according to the instruction provided in the SYBR qPCR Mix kit.

Statistical analysis

Statistical significance were analyzed by Student's *t*-test or one-way ANOVA analysis using SPSS. Three independent experiments were conducted to obtain the data and differences were considered statistically significant when P value <0.05 ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).