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Human mesenchymal stem cells may involve in keloid pathogenesis

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Abstract

Background

Pathogenesis of keloid is unknown. Although vigorous investigations were attempted to elucidate the mechanisms or causative factors on keloid, there are little on why this is very intractable and easily recurrent in each patient.

Methods

In an attempt to analyze the possible interaction between human mesenchymal stem cells and keloid-derived fibroblasts, the dual-chamber cell-migration assay, cell proliferation, ultrastructural morphology and Western blot analysis of the production of the extracellular matrices of the co-culture were investigated.

Results

Cell proliferation was not significantly different between keloid-derived fibroblasts and normal dermal fibroblasts during a 4-day observation period. There was significant cell migration of human mesenchymal stem cells when keloid-derived fibroblasts were placed in the bottom chamber compared to when normal dermal fibroblast were placed in the same way in 8-μm diameter pore membranes (190.6±51.45, 32.0±6.20 cells/field, respectively, p<0.01). With 3-μm diameter pores, the human mesenchymal stem cells migrated in the pores only when the
keloid-derived fibroblasts were placed in the bottom chambers (6.4±3.84 cells/field).

Monolayer co-culture of hMSCs and keloid-derived fibroblasts demonstrated further functional differentiation such as collagen secretion and abundant rough endoplasmic reticulum. Western blot analysis in the modified dual chamber culture demonstrated most significantly abundant fibronectin expression when the human mesenchymal stem cells with keloid fibroblasts than others.

**Conclusion**

These results may indicate that human mesenchymal stem cells participate and recruit in keloid pathogenesis by differentiating themselves toward keloid recalcitrant formation and progression.

**Keywords**

in vitro, migration, keloid, pathogenesis, mesenchymal stem cell, quantitative Western blotting of fibronectin

**Introduction**

Despite extensive researches focused on keloid mechanisms and treatment including the possible involvement of fibrogenic factors such as transforming growth factor-β (TGF-β) (1), insulin-like growth factor-1 (IGF-1) (2), and platelet-derived growth factor (PDGF) (3) as well as
defects in fibrin degradation represented by plasminogen activator inhibitor-1 (PAI-1) (4), it is little known which cell type plays a crucial role in the pathogenesis of keloids. While keloid scars recur in individual patients in the same area, some patients develop keloid scars in multiple areas after very minor trauma. Keloid scars are recalcitrant to medical, radiation-therapeutic, or surgical modalities as well as voluntarily extending over the original wound boundary. There is no fundamental treatment, and current treatment is by palliative modalities such as pressure, anti-allergic medication or steroid injection (5). Recent developments in stem cell biology have led to the potential application of regenerative medicine by stem cells in the wound healing process. Among somatic stem cells, the human bone marrow-derived mesenchymal stem cells (hMSCs) are well characterized by surface markers, cell proliferation, differentiation and regulation (6). Lipid mediators such as cysteinyl leukotrienes are involved in human mesenchymal stem cell differentiation (7). Keloid fibroblasts significantly enhanced the extracellular matrix (ECM) expression such as collagen type 1, fibronectin and PAI-1 under TGF-β and IGF-1 in dose-dependent manner (8). Thus excessive ECM production from keloid fibroblast is targeted of therapy and pathogenesis. One example, a mouse systemic sclerosis model by daily bleomycin injection reversed the sclerotic cutaneous change by the intravenous administration
of antibody against TGF-β (9), however, detailed information concerning the regulation of influx suppression of granulocytes such as mast cells and eosinophils is still unknown. Moreover, systemically administered mesenchymal stem cells able to reverse bleomycin-induced fibrotic effects in lung tissue (10) are a possible therapeutic modality. The interaction between keloid-derived fibroblasts and mesenchymal stem cells has therefore been investigated for cell ultra-structure as well as cell proliferation, cell migration and ECM production.
Materials and Methods

Human mesenchymal stem cells

Human mesenchymal stem cells from a single human bone-marrow donor were isolated by density gradient centrifugation and strictly sorted as positive for markers such as CD105, CD166, CD29 and CD44, and negative for cell surface makers such as CD14, CD34 and CD45. Human mesenchymal stem cells (hMSC) were purchased from BioWhittaker, Inc. (Cat # PT-2501, Walkersville, MD, USA) and the cryopreserved cells were thawed immediately according to the manufacturer’s instructions. Two different donor-derived hMSCs, whose sex are male, whose age are early 20 years (23 years old and 24 years old) and whose racial background are Asian, were used for this study.

The cells were cultured in “basic medium” of Dulbecco’s modified Eagle Medium (DMEM) containing low glucose supplemented with 10% fetal bovine serum (FBS, heat-inactivated, cat # 16000-044, GIBCO, Invitrogen™, Life Technologies, Japan K.K.), 200mM L-glutamine, and penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in 95% humidified air and 5% CO₂. The medium was changed every 3 days until the cells were confluent, and they were then passaged up to 3 times. The growth characteristics during four passages in FBS were indistinguishable.
The cells were washed using 10 ml of phosphate-buffered saline (PBS) and then liberated by exposure to 0.25% trypsin/1 mM EDTA (GIBCO, cat # 25200-056) for three minutes at 37°C, followed by tapping of the dishes and the addition of 5 ml of culture medium. The cells were centrifuged at 400 g, and then re-suspended in basic medium for the following in vitro examinations. The other cells were stored at –70°C until use in a solution containing 5% human serum albumin (IS Japan, Co., Ltd., Saitama, Japan, Cat # 9988) and 10% dimethylsulfoxide (DMSO, Sigma-Aldrich Japan K.K., Tokyo, Japan, Cat # 41641) according to the manufacturer’s instruction.

**Keloid-derived fibroblasts and normal dermal fibroblasts**

Three different human keloid samples were used in this investigation, taken from three different Japanese male patients after surgical excision and subsequent adjuvant electron-radiation therapy. All three male patients ranged from 17 years to 23 years. Informed consent was obtained from each patient. The tissues were divided for tissue samples and cell culture. Some tissues were fixed in ice-cold 4% paraformaldehyde solution for 3 days, embedded in paraffin, and cut into 5 µm-thick sections, and some were prepared for ultra-structural analysis.Slides were stained with hematoxylin and eosin, and Toluidin blue. For cell culture, tissue excised
from a keloid lesion was minced and placed on 100-mm culture plates, consisting of the same “basic medium” as hMSCs at 37°C in 95% humidified air and 5% CO₂ as previously established (2). Normally, keloid-derived fibroblasts can be observed under a phase-contrast microscope after 2-week culture with 3-day medium change. The cells were passaged when 70 to 80% confluence was reached. Up to 5 passages from initial cell dissemination were used in this experiment.

As a control, normal human dermal fibroblasts (CCS-2511), which were oriented from two distinguished male, late teen-age early 20 years (19 years old and 21 years old) and Asian, were cultured using an FGM Bullet kit (CCM-3130), containing 500 ml of fibroblast basal medium supplemented with hFGF-2, insulin, FBS and gentamicin/amphotericin-B (manufactured by Cambrex Corporation, East Rutherford, New Jersey, USA and distributed by Asahi Technoglass Corporation, Tokyo, Japan), on 100-mm culture plates at 37°C in 95% humidified air and 5% CO₂. After 24-hour incubation, the growth medium was changed to “basic medium” in accordance with hMSCs and keloid-derived fibroblasts. The growth medium was changed every other day until the cells were confluent, and they were then passaged up to 3 times. The cells were washed using 10 ml of phosphate-buffered saline (PBS) and then liberated by exposure to 0.25% trypsin/1mM EDTA (GIBCO, cat # 25200-056) for three minutes at
37°C, followed by tapping of the dishes and the addition of 5 ml of culture medium. The cells were centrifuged at 400 g, and then re-suspended in basic medium for the following experiments. The other cells were stored at −70°C until use in a solution containing 5% human serum albumin (IS Japan, Co., Ltd., Saitama, Japan, Cat # 9988) and 10% dimethylsulfoxide (DMSO, Sigma-Aldrich Japan K.K., Tokyo, Japan, Cat # 41641) according to the manufacturer’s instructions.

Cell proliferation

Cell proliferation for keloid-derived fibroblasts and normal dermal cells was determined as previously established (11). In short, 1×10⁴ cells were counted using a Beckman Coulter® Cell and Particle Counter (Beckman Coulter K.K., Tokyo, Japan) in 24-well culture dishes after 24-hour incubation in “basic medium”. All cells were counted in triplicate and the average value was calculated for each well. Cell death was minimal and a trypan blue cell viability assay demonstrated less than 1% non-viable cells throughout the experimental observation period in “basic medium”.

Dual-modified Boyden chamber cell migration assay and co-culture assay

First, cell culture inserts (3.0 μm/6-well or 8.0 μm/6-well) (upper
chamber) initially containing $1 \times 10^5$ hMSCs or normal dermal fibroblasts in basic medium overnight, were placed on the seeded top of a 6-well plate dish (lower chamber) in the same basic medium (12). After overnight incubation, hMSCs or normal dermal fibroblasts attached to the upper chambers were transferred onto each lower chamber, onto which each cell (human keloid-derived fibroblast cells or normal dermal fibroblasts) or medium alone (no cell) was already seeded at about 50% confluence of each 6-well plate in “basic” medium. The migration activity was evaluated 16 hours later by counting the number of invaded hMSCs on the reverse surfaces of 3-μm and -8-μm pore membranes, stained with Nuclear First Red (TA-060-NF) LAB Vision Corporation (Fremont, CA). The experiments were repeated in five different wells for each assay after obtaining the average values of five counts microscopically.

Some hMSCs were then used for direct co-culture in “basic” medium for further direct analysis. In the direct co-culture analysis, $1 \times 10^5$ hMSCs and $1 \times 10^5$ keloid-derived fibroblasts, or $1 \times 10^5$ hMSCs and $1 \times 10^5$ normal dermal fibroblasts were incubated for 24 hours in 6-well plates and subsequently performed for electron microscopic analyses. For each group five different cell culture plates were individually investigated.

Electron microscopy (transmission electron microscopy, TEM and
scanning electron microscopy, SEM)

For transmission electron microscopy (TEM), keloid tissues and basic medium containing hMSCs in 10% FBS were pre-fixed in half-strength Karnovsky fixative (pH 7.2, osmolarity 1,400 mOsm) buffer consisting of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 hours, post-fixed in 2% osmium tetroxide solution (pH 7.4), dehydrated using a conventional procedure and embedded in epoxy resin. The hMSCs were cultured for four days in basic medium of low glucose DMEM supplemented with 10% FBS and 200mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were first centrifuged, and then washed with PBS, and the pellets at the bottom of the tube were subsequently dissolved with fixative after washing with PBS. Embedded specimens were ultrathin-sectioned and double-stained with uranyl acetate and lead citrate. These sections were observed using a Hitachi H-7100 electron microscope (Hitachi, Tokyo, Japan) at 75 kV accelerating voltage.

Next, specimens of the cell culture insert, to which the hMSCs were attached on the reverse side, prepared for scanning electron microscopy (SEM), were dehydrated and dried by critical-point drying apparatus, HCP-2 (Hitachi, Tokyo) for scanning electron microscopy. Specimens in a 35mm tissue culture dish, 3000-035 (IWAKI, Tokyo) were dehydrated through an ethanol series and freeze dried in t-butyl alcohol in a
freeze-dryer. The dried specimens were scatter-coated with gold using an ion-coater, IB-2 (Eiko Engineering, Tokyo) and observed with a scanning electron microscope, S-3500N (Hitachi, Tokyo)

**Western Blotting**

For Western blotting, extracts of $5 \times 10^5$ hMSCs and $5 \times 10^5$ keloid-derived fibroblasts, $5 \times 10^5$ hMSCs and $5 \times 10^5$ normal dermal fibroblasts or $5 \times 10^5$ normal dermal fibroblasts and $5 \times 10^5$ keloid-derived fibroblasts, in the upper and in the lower chamber, respectively, from 8-μm pore-sized dual chamber method, were prepared after 24-hour incubation, using celLytic-M (cat #C2978, Sigma-Aldrich, St. Louis, MO) with aliquots containing 20 μg as protein as measured by spectrophotometer, run on an e-pagel (Atto, Co. Ltd, Tokyo, Japan) sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis system (25 mM Tris, 0.1% SDS and 192 mM glycine solution). Cells for Western blotting were obtained only from each lower chamber. The gels were washed with TBST Solution (20 mMTris-HCl, pH 7.4, 0.5 M NaCl, 0.05% Tween-20) for 3 minutes, three times in a horizontal shaker. The proteins were then transferred to Hybond-P PVDF membrane (RPN2020P, Amersham Pharmacia Biotech, Inc., Piscataway, NJ) in a solution of 25 mM Tris, 20% methanol, 192 mM glycine.
The nonspecific background was blocked with 0.2% bovine serum albumin in PBS for 1 hour in a horizontal shaker. The membranes were incubated for 3 hours with primary antibodies against mouse fibronectin monoclonal antibody (1:200 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, Cam Cat # sc-8422) or mouse monoclonal GAPDH antibody (1:300 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, Cam Cat # sc-47724), and then incubated with secondary antibodies of mouse anti-rabbit HRP conjugated antibody (1:1000 dilution; Amersham Pharmacia Biotech, Inc.). The blotted membranes were washed with TBST solution after each primary or secondary antibody for 3 minutes three times in a horizontal shaker. After incubation with ECL plus (Amersham Pharmacia Biotech Inc.) for 3 minutes, the membranes were visualized in a CCD camera-loaded chemiluminescence system for protein expression (ATTO Light Capture, cat # 6962, ATTO, Tokyo, Japan). Five different samples from different tissues of each group were investigated using Western blot analyses, and the protein levels were densitometrically analyzed using a CS analyzer (Atto). For each band, the mean of five measurements was calculated. The fibronectin expression levels were normalized against internal GAPDH expression levels of each tissue.
**Statistical Analysis**

The cell number values are expressed as the means ± standard deviation. The data among the groups were analyzed by one-way analysis of variance (ANOVA) followed by Scheffe’s F test using add-in software, Statcel, to Microsoft® Excel 2000. P-values less than 0.05 were considered significant.

**Results**

**Cell proliferation**

The keloid-derived fibroblasts proliferated at $2.4 \pm 2.48 \times 10^4$ cells at day 1, 24 hours after plating the cells, then $5.1 \pm 2.07 \times 10^4$ cells at day 2, $13.2 \pm 1.16 \times 10^4$ cells at day 3 and $11.3 \pm 1.34 \times 10^4$ cells at day 4 ($p<0.01$).

The normal dermal fibroblasts proliferated at $3.8 \pm 1.53 \times 10^4$ cells at day 1, then $5.2 \pm 3.11 \times 10^4$ cells at day 2, $7.3 \pm 3.29 \times 10^4$ cells at day 3 and $10.7 \pm 4.58 \times 10^4$ cells at day 4 ($p<0.01$). Both the keloid-derived fibroblasts and normal dermal fibroblasts demonstrated linear cell proliferation during 4-day observation.

**Quantitative cell migration**

The hMSCs were vital and the cells were healthy throughout the
experiment. When normal dermal fibroblasts were placed in the lower chambers and with 3-μm pore membranes, the hMSCs only invaded 0.4 ± 0.5 cells/field. The keloid-derived fibroblasts induced hMSC migration through 3-μm pore membranes at 6.4 ± 3.8 cells/field, while keloid-derived fibroblasts induced 2.4 ± 1.3 cells/field cell migrations of normal dermal fibroblasts. There was a significant difference in hMSC migration with 8-μm membranes among the hMSCs in the upper and normal fibroblasts in the lower, the hMSCs in the upper and the lower and normal dermal fibroblasts in the upper and the keloid-derived fibroblasts in the lower (32.0 ± 6.2, 190.6 ± 51.4, 65.2 ± 9.0 cells/field, respectively, p<0.01) (Figure 1A and 1B). Since the keloid-derived fibroblasts demonstrated significant cell migration, further investigation following cell-to-cell interaction, direct co-culture between the keloid-derived fibroblasts and hMSCs as well as keloid-derived fibroblast analysis was performed.

**Electron Microscopy**

In the dual-cell migration assay, cell morphology in the electron microscope demonstrated the differentiation of cells, such as conspicuous nucleoli and cytoplasmic sub-cytomembranous fibrils with dense bodies indicating myofibroblast-like structure in some cells, and thick collagen secreted granules as well as frequent mitoses (Figure 2). Subsequently,
monolayer co-culture of hMSCs and keloid-derived fibroblasts demonstrated further functional differentiation such as collagen secretion and abundant rough endoplasmic reticulum (Figure 3).

**Western blotting and quantification of extracellular matrix**

The western blot analysis of either the hMSCs in the upper chamber or none and either normal dermal fibroblasts or keloid-derived fibroblasts in the lower chamber for 24 hours demonstrated significant difference in the fibronectin expression. Normalized by internal GAPDH expression for each band, the hMSCs in the upper and normal dermal fibroblasts depicted 0.8 ± 0.2, the hMSCs in the upper and keloid-derived fibroblasts depicted 2.2 ± 0.4 and normal dermal fibroblasts in the upper and keloid-derived fibroblasts in the lower depicted 1.0 ± 0.2 relative value of fibronectin expression (p<0.01, between hMSCs in the upper with normal fibroblasts in the lower and hMSCs in the upper with keloid-derived fibroblasts in the lower, between hMSCs in the upper with keloid-derived fibroblasts in the lower and normal fibroblasts in the upper with keloid-derived fibroblasts in the lower)(Figure 4A and 4B)
Discussion

Keloid-derived fibroblasts proliferated in the “basic” medium of hMSCs during 4-day observation. There was little cell apoptosis during the experimental period. The dual chamber assay demonstrated that hMSCs significantly induced cell migration with 8-μm pore membranes when keloid-derived fibroblasts were placed in the bottom plates compared to normal dermal fibroblasts in the bottom plates. In addition, even 3-μm pores, which normally do not permit the passage of hMSCs, were able to migrate. The significant cell migration suggests that keloid-derived fibroblasts are able to induce hMSC chemoattraction toward keloid cells. Normal fibroblast failed to be chemoattracted to keloid-derived fibroblasts.

In the next step, to clarify either cell-to-cell or humoral interaction with keloid-derived fibroblasts, ultrastructural analyses with both a 3-μm pore membrane and direct monolayer co-culture demonstrated hMSC cellular changes toward cell differentiation, especially cytoplasmic structural changes toward myofibroblasts. Collagen fiber bundles were expressed and secreted in the dual-chamber assay and the cell morphology changed even more to myofibroblasts with abundant myofibers, rough endoplasmic reticulae and the secretion of collagen bundles. As a feature of predominant “myofibroblasts” in the keloid (13), our data supported these characteristics as well as the invasion observed in IGF-I pathways (2) observed in the cell
migration assay. Taken together, hMSCs are able to migrate into sites where keloid fibroblasts exist and may contribute to keloid pathogenesis as demonstrated by rich collagen production in the cytoplasms of hMSCs observed in both dual and direct co-culture systems. The hMSCs are able to interact with other cells in vitro as previously demonstrated in a dual-chamber migration study (12), and further this study confirmed that the hMSCs are able to induce extracellular matrices when co-cultured with keloid fibroblasts. Also this is the first evidence that keloid fibroblasts or keloid-derived humoral factors induced the hMSC differentiation although lipid mediators demonstrated the hMSC differentiation (7). Keloid-derived fibroblasts demonstrated significantly abundant fibronectin expression with hMSCs in the upper chamber. This suggests that hMSCs may participate in the keloid site and produce more extracellular matrices and thus may cause extend the keloid formation and also exaggeration of keloid. These may be implicated in the recurrence of keloids in the same area or the exacerbation of pre-existing keloids through the blood stream since good reproducible animal models are established yet for investigation of keloid recurrence and extension.
Figure legends

Figure 1: Cell migration in the reverse side of 3-μm pore membranes when keloid-derived fibroblasts were placed in the lower chamber in the dual-chamber assay.

There was significant cell migration when keloid-derived fibroblasts were placed in the bottom chamber compared to the use of normal dermal fibroblasts (p<0.01) and 8-μm pore membranes. hMSCs were able to pass through even 3-μm pore membranes when keloid-derived fibroblasts were used but very few with normal dermal fibroblasts. More than 10-μm-diameter hMSCs were able to pass through the 3-μm diameter pores.

Figure 2: The ultrastructures of hMSCs when keloid-derived fibroblasts were placed in the lower chamber in the dual-chamber cell migration assay.

The reverse side of transmission electron microscopy (TEM) demonstrated ultrastructural differences among the treatments.

A: Conspicuous nucleoli in the elongated nuclei and microfilaments with dense bodies indicating contractive ability are found in the cell periphery. The cytoplasm developed a rough endoplasmic reticulum (×5,000).
B: The high-power view demonstrated evident microfilaments with dense bodies of contractile filaments similar to those observed in myofibroblasts or smooth muscle cells (×10,000).

Figure 3: Monolayer co-culture of hMSCs and keloid-derived fibroblasts (×10,000).

The direct co-culture of keloid-derived fibroblasts and hMSCs demonstrated more progressed myofibroblastic changes, characterized by cytoplasmic differentiation as depicted by abundant rough endoplasmic reticulum and the secretion of collagen-like fibers as well as actin-type microfilament bundles.

Figure 4: Fibronectin Western blot analysis from the dual chamber incubation for 24 hours.

A: Blotting demonstrated each combination of the cells (top) and internal GAPDH expression (bottom). The extracts were obtained from the bottom plates.

B: Densitometric analysis of each combination of the cells which is normalized by internal GAPDH expression. Five-time calculation of each
band densitometry was statistically compared. When hMSCs in the upper and keloid-derived fibroblasts in the lower chamber, there were significant differences compared to the others (p>0.01).
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Keloid-like changes of hMSCs

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Figure 1B

**Quantitative cell migration**

- **hMSC/N**: hMSCs in the upper and normal dermal fibroblasts in the lower chamber
- **hMSC/K**: hMSCs in the upper and keloid-derived fibroblasts in the lower chamber
- **N/K**: normal dermal fibroblasts in the upper and keloid-derived fibroblasts in the lower chamber

****: $p<0.01$ to both hMSC/N 8 μm and N/K 8 μm

**: $p<0.01$ to each 3 μm
Figure 2B \times 10,000
1 hMSCs in the upper and normal dermal fibroblasts in the lower chamber
2 hMSCs in the upper and keloid-derived fibroblasts in lower chamber
3 normal dermal fibroblasts in the upper and keloid-derived fibroblasts in the lower chamber
Figure 4B

**Densitometry of Western blotting**

- **hMSC/N**: hMSCs in the upper and normal dermal fibroblasts in the lower normalized by GAPDH
- **hMSC/K**: hMSCs in the upper and keloid-derived fibroblasts in the lower normalized by GAPDH
- **N/K**: normal dermal fibroblasts in the upper and keloid-derived fibroblasts in the lower normalized by GAPDH

****: p < 0.01 to both hMSC/N and N/K