Transdifferentiation of Peripheral Blood Mononuclear Cells into Epithelial-Like Cells

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Bone marrow-derived stem cells have the potential to transdifferentiate into unexpected peripheral cells. We hypothesize that circulating bone marrow-derived stem cells might have the capacity to transdifferentiate into epithelial-like cells and release matrix metalloproteinase-1-modulating factors such as 14-3-3 σ for dermal fibroblasts. We have characterized a subset of peripheral blood mononuclear cells (PBMCs) that develops an epithelial-like profile. Our findings show that these cells develop epithelial-like morphology and express 14-3-3 σ and keratin-5, -8 as early as day 7 and day 21, respectively. When compared with control, conditioned media collected from PBMCs in advanced epithelial-like differentiation (cultures on days 28, 35, and 42) increased the matrix metalloproteinase-1 expression in dermal fibroblasts ($P \leq 0.01$). The depletion of 14-3-3 σ from these conditioned media by immunoprecipitation reduced the effect by 39.5% (P value, 0.05). Therefore, the releasable 14-3-3 σ from PBMC-derived epithelial-like cells is involved in this process. Our findings provide new insights into the PBMC transdifferentiation to generate epithelial-like cells and subsequently release of 14-3-3 σ that will disclose new therapeutic alternatives for different dermal clinical settings. (Am J Pathol 2007, 171:1140–1152; DOI: 10.2353/ajpath.2007.070051)

Adult stem cells have the potential of self-renewal and terminal differentiation to replace peripheral mature cells continuously lost because of normal tissue turnover.¹ Although numerous articles have identified adult stem cells in tissues such as skin, fat, muscle, blood vessels, and brain, among others, the hematopoietic tissue (from bone marrow and peripheral blood) represents one of the most extensively studied in terms of its cell dynamism and heterogeneity.^{2–4}

For instance, 20 to 100 hematopoietic stem cells have the capacity to replace the whole lymphohematopoietic system in adult mice after lethal doses of radiation.⁵ The percentage of donor cell engraftment can also be assessed in skin, esophagus, stomach, small bowel, large bowel, and bronchi.^{6,7} Other studies have reported similar results in kidney epithelium, pancreas, myocardium, skeletal muscle, and central nervous system neurons.^{7–10} Bone marrow-derived cells have been reported as transit-amplifying cells at the injured tissue, where they differentiate into keratinocytes.¹¹ Clinically, in a study of archival specimens from patients who received transplantation of peripheral-blood stem cells, Korbiling and colleagues¹² demonstrated that those cells could differentiate into mature epithelial cells in skin, lungs, gastrointestinal tract, and liver. In addition, other authors have described myocardial regeneration,^{13–15} and neuron renewal.¹⁶ In similar experiments involving skin damage, the application of CD34⁺ peripheral blood mononuclear cells (PBMCs) accelerated the neovascularization and epidermal healing in a model of chronic full-thickness skin wounds in diabetic mice.¹⁷ A similar clinical approach has been performed in chronically wounded patients by using autologous bone marrow-derived cells applied on the wound surface and injected into the wound margins. As a result of this treatment, patients healed completely, and dermis tended to recover its structure.¹⁸ Therefore, the traditional concept of a hierarchical hematopoietic stem cell differentiation with a restrictive, unidirectional, and preprogrammed cell commitment has been changed for a more flexible and reversible system.^{1,7,13,19} Thus, adult stem and precursor cells can move through boundaries of cell lineage, tissue, and germ layer to give rise to unexpected nonhematopoietic cells according to the tissue in which they reside, especially after inductive signals such as tissue damage, transplantation, or in ex vivo culture.^{1,7,13,19–21} This phenomenon, called cell transdifferentiation, lineage con-

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version, or stem cell plasticity, constitutes an important characteristic of bone marrow-derived cells to repopulate somatic tissues.⁵ Thus, circulating bone marrow-derived hematopoietic stem cells (also referred to as CD34⁺ cells) migrate to damaged areas and participate in the local tissue regeneration.^{1,7,19–21} Thus, circulating CD34⁺ bone marrow-derived adult stem cells constitute fibrocytes that rapidly infiltrate the wound bed and settle in particular locations within the dermis.^{22–25} Subsequently, the exposure to transforming growth factor- β induces the fibrocyte differentiation into myofibroblasts and could contribute to the tissue contraction.^{26–28} In addition, within resident stem cell niches of skin, located at the bulge region of hair follicles and basal layer of the interfollicular epidermis, CD34⁺ keratinocyte precursors have been identified.^{11,29} Even though the local injection of CCL27 seems to accelerate healing process by increasing the CD34⁺ bone marrow-derived cell migration,³⁰ the source of these cells as well as their role in both normal cell renewal and tissue repair have yet to be determined. Unfortunately, there is no reliable marker that displays long-term expression for local identification and tracking of cells along the transition process of epithelial cell maturation. In this regard, the protein 14-3-3 σ has been described as a specific marker for epithelial cells.^{31,32} In addition, we have recently described a releasable form of keratinocyte-derived 14-3-3 σ that induces matrix metalloproteinase (MMP)-1 expression in dermal fibroblasts.³³ Thus, because of its distinct expression pattern and anti-fibrotic effects on dermal fibroblasts, 14-3-3 σ serves as a promising protein to elucidate further the functional commitment of PBMCs into epithelial-like cells.

To assess our working hypothesis that circulating bone marrow-derived stem cells (within PBMCs) might transdifferentiate into epithelial-like cells, series of experiments were conducted, the findings of which are presented here. To characterize further the functionality of these epithelial-like cells, the release of 14-3-3 σ and its effect on MMP-1 expression in dermal fibroblasts were examined.

Materials and Methods

Isolation of PBMCs

PBMCs were isolated from whole blood of volunteers by using Ficoll-Hypaque density gradient centrifugation following the manufacturer's protocol. Blood from donors was carefully layered on Ficoll solution (density 1.077; Sigma-Aldrich, St. Louis, MO). Centrifugation was performed at $931 \times g$ for 20 minutes at 20°C (Centrifuge Allegra X-12R; Beckman Coulter, Inc., Palo Alto, CA). The mononuclear cell interphase was taken and washed three times in $1 \times$ phosphate-buffered saline (PBS) for 8 minutes at $300 \times g$ (Centrifuge Allegra X-12R; Beckman Coulter, Inc.). Cells were counted and suspended in appropriate culture medium (see below) containing 2×10^6 cells/ml so that cell suspensions were added to either chamber slides (Lab-Tek II; Nalge Nunc International, Naperville, IL) or 75-cm² flasks (BD Falcon; BD Biosciences, Bedford, MA).

Isolation of Human Circulating Precursor Cells from PBMCs

After PBMCs were isolated, circulating stem/precursor cells were isolated by using the EasySep negative selection human progenitor cell enrichment kit with CD41 depletion (StemCell Technologies, Vancouver, BC, Canada) following the manufacturer's recommendations. Cell suspension at a concentration of 5×10^7 cell/ml were prepared using $1 \times$ PBS containing 2% heat-inactivated fetal bovine serum qualified (FBS; Gibco-Invitrogen Corp., Grand Island, NY). Within a 12×75 -mm polystyrene tube, the EasySep negative selection progenitor cell enrichment cocktail with CD41 depletion was added at 50 μ l/ml cell suspension. The mixture was incubated for 15 minutes at room temperature. Then, EasySep magnetic nanoparticles (StemCell Technologies) were added at 50 μ l/ml cell suspension and incubated at room temperature for another 15 minutes under constant rotation. After mixing, the sample tube was placed without cap into the magnet (EasySep Magnet 18000; StemCell Technologies) for 10 minutes. The liquid content with wanted cells was subsequently poured off into a new tube.

Culture of PBMCs and Circulating Precursor Cells and Stimulation of Epithelial-Like Cell Transdifferentiation

After the isolation procedure, PBMCs were resuspended in culture medium containing 49% Dulbecco's modified Eagle medium (DMEM; Gibco-Invitrogen), 49% defined keratinocyte serum-free medium (KSFM) (Gibco-Invitrogen Corp.), 2% FBS (Gibco-Invitrogen Corp.), growth supplements (Gibco-Invitrogen Corp.), penicillin G sodium (100 U/ml), and streptomycin sulfate (100 μ g/ml), and amphotericin B (0.25 μ g/ml) (Gibco-Invitrogen Corp.). Half of the culture medium was changed every other day. At different time points, cells were harvested by using 0.05% ethylenediaminetetraacetic acid (EDTA) and 0.1% trypsin (Gibco-Invitrogen Corp.) in $1 \times$ phosphate-buffered saline (PBS) and gentle scraping with a rubber policeman. In case of morphological studies, adherent cells were directly fixed on chamber slides with 4% paraformaldehyde. In parallel experiments, at the same time points, conditioned media from PBMC-derived epithelial-like cells were collected after a 24-hour incubation with a test media containing 50% DMEM and 50% KSFM without supplement and growth factors. The same procedure described above was used with circulating precursor cells to induce cell transdifferentiation into epithelial-like cells.

Clinical Specimens and Cell Culture

After voluntary recruitment and informed consent, foreskin samples were obtained from infants undergoing elective circumcision, under local anesthesia, according to a protocol approved by the Clinical Research Ethics Board, Office of Research Services of the University of

British Columbia. Skin samples were collected individually and washed several times in sterile 1× PBS supplemented with antibiotic-antimycotic preparation (100 µg/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B) (Gibco-Invitrogen Corp.). Epidermal and dermal layers were separated by treatment with 40 mg/ml dispase (Gibco-Invitrogen Corp.) during 2 hours at 37°C. Culture of fibroblasts were established as previously described.³⁴ When reaching confluence, fibroblasts were detached by trypsinization and subculture into 75-cm² flasks (BD Falcon, BD Biosciences). Fibroblasts at passages 3 to 7 were used in co-culture system. To establish keratinocyte cultures, epidermis was treated separately in 0.05% EDTA/0.1% trypsin (Gibco-Invitrogen Corp.) at 37°C for 5 minutes to release basal keratinocytes. After neutralization with DMEM and 10% FBS and washing three times with 1× PBS, cells were cultured in KSFM (Gibco-Invitrogen Corp.) supplemented with BPE (50 µg/ml), and epidermal growth factor (5 µg/ml). Keratinocytes from passages 3 to 5 were used as positive control after inducing cell maturation with DMEM and 2% FBS.

Epithelial-Like Cells/Dermal Fibroblasts Co-Culture System

To identify the effect of releasable factors of PBMC-derived epithelial-like cells on dermal fibroblasts, PBMCs were cultured in 30-mm-diameter culture plate inserts (Millicell-PCF; Millipore Corp., Billerica, MA). At different time points (days 0, 7, 14, 21, 28, 35, 42, and 49), these inserts were placed into six-well plates containing subconfluent culture of dermal fibroblasts by using 2 ml of test media prepared with 49% DMEM and 49% KSFM plus 2% FBS with no other additives. Only releasable factors from PBMC-derived epithelial-like cells (upper chamber) can pass through the permeable membrane of inserts (0.4-µm pore size) and interact with dermal fibroblasts (lower chamber). As a negative control, a co-culture system between fibroblasts located on inserts (upper chambers) and fibroblasts located in lower chambers was used. As a positive control, differentiated keratinocytes were used on inserts (upper chambers). After a 24-hour incubation, dermal fibroblasts from lower chambers were harvested by using 0.05% EDTA and 0.1% trypsin in 1× PBS and gentle scraping with a rubber policeman. After three washing steps with 1× PBS, fibroblast lysates were obtained with cell lysis buffer [50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA (Sigma), 1 mmol/L EGTA (Sigma), 0.025% NaN₃, 1% Triton X-100, 0.5% Igepal CA-630 (Sigma), and protease inhibitor cocktail (Sigma)]. Protein concentrations were measured by using the Bradford method with a Life Science UV/Vis spectrophotometer (DU 530; Beckman Coulter, Inc.). To determine the effect of PBMC-derived epithelial-like cell-released factors on expression of MMP-1 by dermal fibroblasts, total protein content was extracted from fibroblasts grown in lower chambers and MMP-1 expression was evaluated by Western blotting.

Immunocytochemistry

At different time points, cells cultured on chambered slides were fixed in 4% paraformaldehyde for 10 minutes followed by incubation in 1× PBS within a staining chamber for 5 minutes at room temperature. To inactivate endogenous peroxidase, slides were incubated with 30% hydrogen peroxide (Sigma-Aldrich) diluted in methanol (2% v/v) for 15 minutes. After washing twice with ddH₂O and rehydration with 1× PBS for 5 minutes, samples were incubated in blocking solution (1× PBS containing 10% goat serum plus 5% bovine serum albumin; Sigma) within a humidified chamber for 30 minutes. The primary antibody was then applied and incubated inside of a humidified chamber overnight at 4°C. Two epithelial markers were selected to demonstrate cell transdifferentiation from PBMCs into epithelial-like cells. Thus, rabbit anti-human 14-3-3σ (a kind gift of Dr. A. Aitken, University of Edinburgh, Edinburgh, UK) and mouse anti-human keratin-5, -8 monoclonal antibodies (Chemicon International, Temecula, CA) at 1:1000 dilution were used. After rinsing three times with 1× PBS for 5 minutes each, samples were incubated with either biotinylated goat anti-rabbit IgG (lot BA-1000; Vector Laboratories Inc., Burlingame, CA) or goat anti-mouse IgG (lot BA-9200; Vector Laboratories Inc.) at 6 µg/ml for 45 to 60 minutes at room temperature. Application of StreptoAB-Complex/horseradish peroxidase subsequently was performed according to the manufacturer's recommendations (DakoCytomation, Glostrup, Denmark). Samples were then exposed to peroxidase substrate solution [25 mg of 3,3'-diaminobenzidine (Sigma), 50 ml of 1× PBS, and 50 µl of 30% H₂O₂] for 5 minutes. In addition, counterstaining with hematoxylin was performed using a standard protocol. Finally, dehydration with graded ethanol and treatment with xylene were performed before the application of Permount (Fisher Scientific, Fair Lawn, NJ) and coverslips. Images were obtained using a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Toronto, ON, Canada) with Northern Eclipse image analysis software (Empix Imaging, Inc., Mississauga, ON, Canada).

Immunofluorescence and Confocal Microscopy

Cultured epithelial-like cells were fixed in 2% paraformaldehyde for 10 minutes and then washed in 1× PBS and graded ethanol. Nonspecific bindings were avoided by a blocking solution (1× PBS containing 10% goat serum and 5% bovine serum albumin; Sigma). For immunofluorescence microscopy, dual staining was performed using primary rabbit anti-human 14-3-3σ antibodies (kindly provided by Dr. A. Aitken, University of Edinburgh) and mouse anti-human keratin-5, -8 monoclonal antibodies (Chemicon International) at 1:500 dilution and incubated overnight in a humidified chamber at 4°C. After three washing steps with PBS-Tween 20 for 5 minutes each, samples were incubated with rhodamine-conjugated goat anti-rabbit IgG (Chemicon International) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Chemicon International) at 1:3000 dilution for 45 minutes in dark condition. Finally, after washing with PBS-

Tween 20 three times for 5 minutes each, samples were mounted in Vectashield H-1200 (Vector Laboratories, Inc.) containing 4,6-diamidino-2-phenylindole (DAPI) for nuclei staining. A Zeiss Axioplan 2 microscope and Northern Eclipse image analysis software were also used to obtain the images. In addition, single staining was conducted to identify the presence of CD34 (catalog no. 555820; Pharmingen, BD Biosciences, San Jose, CA) and involucrin (catalog no. I9018; Sigma) at 1:1000 dilution each. In both cases, FITC-conjugated goat anti-mouse IgG (Chemicon International) was used as secondary antibody as described above. In confocal microscopy, similar procedures were performed. However, a cocktail of purified mouse anti-human monoclonal cytokeratin-14, -15, -16, and -19 antibodies (catalog no. 550951; Pharmingen, BD Biosciences) at 1:1000 dilution was used instead of mouse anti-human keratin-5, -8 monoclonal antibody. A Zeiss Axiovert 200 microscope and LSM510 META analysis software were also used to obtain the images. To study intracellular details of cytokeratins, the deconvolution procedure with Volocity software (Improvision, Coventry, UK) was used. This technique removes the out-of focus information from the image plane of interest.

Enzyme-Linked Immunosorbent Assay for 14-3-3 σ Detection in Conditioned Media

To determine the amount of soluble 14-3-3 σ protein in conditioned media from PBMCs at different stages of differentiation into epithelial-like cells, an enzyme-linked immunosorbent assay (ELISA) was performed. Briefly, each sample of conditioned media was diluted 50/50 using 5 $\times$ coating buffer (0.1 mol/L NaHCO₃, pH 9.3). Then, 96-well plates (pretreated ELISA plate, Nunc-Immuno Plate MaxiSorp Surface; Nalge Nunc Int.) were coated with 100 μ l per well of diluted samples and incubated overnight at 4°C. After washing twice with distilled/deionized water (150 μ l per well), wells were blocked by adding 2.5% bovine serum albumin (crystallized; Sigma Chemical Co.) for 1 hour at 37°C. After removing the blocking solution, mouse anti-human 14-3-3 σ monoclonal antibody (catalog no. MS-1185-P; NeoMarkers, Fremont, CA) was added at 1:1000 dilution with PBS-Tween 20 (PBS-T) plus 0.5% bovine serum albumin and incubated 1 hour at 37°C. After washing three times with PBS-T, peroxidase affinity rabbit anti-mouse IgG (H+L) (catalog no. 315-035-003; Cedarline; Hornby, BC, Canada) was added in 0.5% bovine serum albumin/PBS at 100 μ l per well for 45 minutes at 37°C. Finally, after removing the secondary antibody and washing wells three times with PBS-T, 50 μ l per well of substrate solution (0.4 mg/ml o-phenylenediamine in 0.05 mol/L phosphate-citrate buffer, pH 5.0, plus 40 μ l of 30% H₂O₂ per 100 ml of substrate buffer solution; Sigma) was added according to manufacturer's recommendations and incubated at 37°C. The optical density of each well was measured by using an ELISA reader (VERSAmax; Molecular Devices, Sunnyvale, CA) and the software SOFTmax Pro. A standard curve was designed with serial dilutions of recom-

binant 14-3-3 σ protein (0, 0.15, 0.31, 0.62, 1.25, and 2.5 ng/ μ l). ELISA from each sample was performed in triplicate. To measure tumor necrosis factor- α and interleukin-1 in PBMC-conditioned media at different time points of epithelial-like cell differentiation, ELISA was also performed. In these cases, each sample of conditioned media was diluted 1:5 in 1 $\times$ coating buffer. As primary antibodies, mouse anti-human tumor necrosis factor- α , clone 2C8 (catalog no. MAB1021; Chemicon International) at 1:500 dilution and rabbit anti-human interleukin-1 α (catalog no. AB1414; Chemicon International) at 1:500 dilution were used. As secondary antibodies, peroxidase affinity rabbit anti-mouse IgG (H+L) (catalog no. 315-035-003; Cedarline) at 1:5000 dilution and peroxidase affinity goat anti-rabbit IgG (H+L) (catalog no. 111-035-003; Cedarline) at 1:5000 dilution were used, respectively. Recombinant human tumor necrosis factor- α (catalog no. 554618; Pharmingen, BD Biosciences) and recombinant human interleukin-1 α (catalog no. IL001; Chemicon International) were used to establish serial dilutions of corresponding standard curves.

14-3-3 σ Immunoprecipitation

The 14-3-3 σ immunoprecipitation was designed to demonstrate the effect of 14-3-3 σ released by PBMC-derived epithelial-like cells on MMP-1 expression in dermal fibroblasts. PBMC-conditioned media from day 28 of cell differentiation into epithelial-like cells were selected to be used (see below). Thus, 1 ml of media from three independent experiments without any supplement or growth factors were incubated with mouse anti-human 14-3-3 σ antibody (catalog no. MS-1185-P; NeoMarkers) for 1 hour at room temperature and under constant rotation. Then, 100 μ l of Protein G (Protein G-Sepharose 4 Fast Flow, catalog no. 17-0618-01; GE Health Care, Uppsala, Sweden) was added to each tube, and a second incubation was performed for 1 hour at room temperature. Finally, tubes were centrifuged at 5000 $\times g$ for 1 minute. 14-3-3 σ -depleted supernatants were therefore obtained to treat three different strains of dermal fibroblasts and compared with full-content PBMC-conditioned media from the same time points. Recombinant 14-3-3 σ was prepared according to a previously described method,³⁵ and used to treat dermal fibroblasts at 4 μ g/ml (positive control). In addition, fibroblasts treated with culture medium without any supplement and growth factors were used as a negative control.

Western Blot Analysis

To evaluate the expression of 14-3-3 σ protein, cell lysates from PBMC-derived epithelial-like cells at different time points of differentiation (days 0, 7, 14, and 21) were obtained. Initially, cells were washed with 1 $\times$ PBS and harvested with 0.05% EDTA and 0.1% trypsin (Gibco-Invitrogen Corp.) in 1 $\times$ phosphate-buffered saline (PBS) and gentle scraping with a rubber policeman. After three washing steps, cell lysates were obtained with cell lysis buffer [50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA (Sigma), 1 mmol/L EGTA (Sigma), 0.025%

Figure 1. Morphology and immunocytochemistry of PBMCs at different time points of cell differentiation. **A:** Morphological changes and immunocytochemical expression of 14-3-3 σ on PBMCs at different time points of cell differentiation. Rabbit anti-human 14-3-3 σ and rabbit nonimmune IgG (negative controls) were used as primary antibodies. Then, samples were incubated with goat anti-rabbit biotinylated secondary antibody and subsequently with StreptoABComplex/horseradish peroxidase. Finally, 3,3'-diaminobenzidine (DAB) was used as peroxidase substrate solution. Cells show 14-3-3 σ staining from day 7 in culture. **B:** Immunocytochemical expression of keratin-5, -8 on PBMCs at different time points of cell differentiation is shown. Mouse anti-human keratin-5, -8 and mouse nonimmune IgG (negative controls) were used as primary antibodies. Then, samples were incubated with goat anti-mouse biotinylated secondary antibody and subsequently developed as described above. Cells show keratin-5, -8 staining from day 21 in culture. To test the expression of 14-3-3 σ and keratin-5, -8, fibroblasts were used as negative control and keratinocytes as positive control (**C** and **D**, respectively). Scale bars: 50 μ m (**A**); 100 μ m (**B-D**).

NaN₃, 1% Triton X-100, 0.5% Igepal CA-630 (Sigma), and protease inhibitor cocktail (Sigma)]. Levels of protein content were determined, and equal amounts (20 μ g per sample) of total protein were fractionated by 12% sodium dodecyl sulfate-polyacrylamide gels and transferred onto Immobilon-P transfer membrane (pore size 0.45 μ m; Millipore Corp.). Nonspecific binding sites on membranes were blocked with 5% skim milk powder in PBS-0.05% Tween 20 overnight at 4°C. Immunoblotting was performed using rabbit anti-human 14-3-3 σ antibody at 1:10,000 dilution for 90 minutes at room temperature. Membranes were then washed four times with Tris-buffered saline containing 0.05% Tween 20 (5 minutes each) and incubated with a 1:3000 dilution of goat anti-rabbit IgG (H+L) horseradish peroxidase-conjugated antibody (Bio-Rad, Hercules, CA) for 90 minutes at room temperature. After washing four times, membranes were incubated with Western blotting luminol reagent according to the manufacturer's recommendations (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and exposed to Kodak BioMax MR Films (Eastman Kodak Co., Rochester, NY) to visualize immunoreactive proteins. As a control for loaded protein amounts, immunoblots were performed with mouse monoclonal anti- β -actin antibody (Sigma) at 1:10,000 dilution followed by a 1:3000 dilution of goat anti-mouse IgG (H+L) horseradish peroxidase-conjugated (Bio-Rad) for 90 minutes at room temperature each. To determine the MMP-1 expression in dermal fibroblasts, we followed the same method as above by using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the primary antibody anti-h-MMP-1 purified mouse monoclonal IgG1 (R&D Systems) at 1:250 dilution and the secondary antibody goat anti-mouse IgG (H+L)-horseradish peroxidase conjugate (Bio-Rad) at 1:2000 dilution.

Statistical Analysis

Nonparametric tests were used to determine the statistical significance of the results. Specifically, the Mann-Whitney rank sum test and the Kruskal-Wallis test were performed to evaluate two independent sample medians and multiple sample groups, respectively. A post hoc Dunn's multiple comparisons test was applied to evaluate further differences among groups. To process data in this report, Microsoft Excel 2000 and SPSS 11.1 (SPSS, Inc., Chicago, IL) for Windows were used. Results are presented as mean $\pm$ SD, where a $P \leq 0.05$ was considered statistically significant.

Results

PBMCs Transdifferentiate into Epithelial-Like Cells

To study PBMC transdifferentiation into epithelial-like cells, we first focused our attention on cell morphology. In this regard, a PBMC subset survived in our established culture conditions and started to increase in size and change their contour. Thus, round-shaped cells

Figure 2. 14-3-3 σ expression in PBMCs at different time points of epithelial-like differentiation. Western blot and densitometric analysis of 14-3-3 σ expression on PBMCs at different time points of cell differentiation into epithelial-like cells. **A:** Western blot of 14-3-3 σ expression in PBMCs on day 0, 7, 14, and 21 in culture. **B:** Densitometric analysis of four different experiments and values are expressed as 14-3-3 σ / β -actin ratio. Statistical significance were reached on days 7 (* P value, 0.02), 14 (** P value, 0.02), and 21 (*** P value, 0.05) compared with undifferentiated PBMCs (day 0).

(PBMCs) with 6- to 10- μ m diameter modified into a transitory state consisting of spindle-like or stellate cells at day 7 in culture and finally into polygonal cells with 180- to 200- μ m size and larger elliptical nuclei at day 21 in culture (Figure 1). To demonstrate the progressive epithelial determination of PBMCs, two specific markers were selected (keratin-5, -8 and 14-3-3 σ) and tested at different time points of epithelial-like cell differentiation. As shown in Figure 1, A and B (days 14 and 21), the morphology of these cells become very similar to those shown for keratinocytes (Figure 1, C and D). Furthermore, along with the increasing size and structural changes toward a keratinocyte-like morphology, PBMCs started to express 14-3-3 σ at day 7 and keratin-5, -8 at day 21 in culture with the defined culture medium (Figure 1, A and B, respectively). The staining pattern of 14-3-3 σ and keratin-5, -8 in these cells on day 21 is very similar to those of positive control (keratinocytes) shown in Figure 1, C and D. Because 14-3-3 σ exerts an extracellular function as an anti-fibrogenic factor, findings from immunocytochemistry were confirmed with Western blot analysis (Figure 2). Thus, when compared with the original undifferentiated PBMCs (day 0), an increased expression of 14-3-3 σ was documented at days 7, 14, and 21 in culture with the established culture condition (Figure 2A). These results correspond to four independent experiments and exhibit statistical significances with P values of 0.02, 0.02, and 0.05, respectively (Figure 2B). These findings have also been confirmed with immunofluorescence microscopy. In this case, the expression of epithelial markers in later stages of epithelial-like transdifferentiation (PBMC days 21, 28, 35, and 42 in established culture conditions) was studied by using

Figure 3. Immunofluorescence microscopy of PBMCs during late stages of epithelial-like cell differentiation. For detection of 14-3-3 σ and keratin-5, -8 expression in PBMCs on days 21, 28, 35, and 42 in culture, PBMCs were fixed in 2% paraformaldehyde for 10 minutes and then washed in 1 $\times$ PBS and graded ethanol. Nonspecific bindings were avoided by a blocking solution (1 $\times$ PBS containing 10% goat serum and 5% bovine serum albumin) for 30 minutes. Rabbit anti-human 14-3-3 σ and mouse anti-keratin-5, -8 monoclonal antibodies were used as primary antibodies at 1:500 dilution and incubated for 1 hour. Then, after three washings with PBS-Tween 20 for 5 minutes each, samples were incubated with rhodamine-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG at 1:3000 dilution for 45 minutes in dark condition. Finally, samples were mounted in Vectashield mounting medium with DAPI for nuclei staining. **A:** Both markers exhibited strong and stable expression during late stages of PBMC differentiation into epithelial-like cells. **B:** Differentiated keratinocytes were used as positive control and dermal fibroblasts as negative control. Scale bars = 100 μ m.

dual staining with 14-3-3 σ (rhodamine) and keratin-5, -8 (FITC). As shown in Figure 3A, these markers exhibit a long-term co-localization within PBMC-derived epi-

thelial-like cells. This finding indicates that PBMCs have the potential to transdifferentiate into keratin-5, -8- and 14-3-3 σ -expressing cells, and this cell profile is

Figure 4. Confocal and immunofluorescence microscopy of circulating precursor cell transdifferentiation into epithelial-like cells. **A:** 14-3-3 σ and cytokeratin-14, -15, -16, and -19 expression in differentiated keratinocytes and circulating precursor cells on day 21 in culture was evaluated by using rabbit anti-human 14-3-3 σ and mouse anti-cytokeratin-14, -15, -16, and -19 as primary antibodies at 1:500 dilution and incubated for 1 hour. After washing, rhodamine-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG were used as secondary antibodies at 1:3000 dilution and incubated for 45 minutes in dark condition. Samples were mounted in Vectafield mounting medium with DAPI for nuclei staining. Deconvolution technique was used to show details of cytokeratin's filament distribution (higher magnification, right-side pictures). Keratinocytes displayed a trabecular pattern of cytokeratins, which co-localized with 14-3-3 σ . Even though similar 14-3-3 σ /cytokeratin co-localization was found, epithelial-like cells exhibited a more homogeneous and denser cytokeratin network that fills the whole cytoplasm. **B:** Single expression of either CD34 or involucrin in circulating precursor cells is shown. A comparison between circulating precursor cell day 1 and day 21 in our established culture condition is made. As primary antibody, either purified mouse anti-human CD34 or monoclonal mouse anti-human involucrin at 1:1000 dilution was used. FITC-conjugated goat anti-mouse IgG was used as secondary antibody at 1:3000 dilution and incubated for 45 minutes in dark condition. Samples were mounted in Vectafield mounting medium with DAPI for nuclei staining. Circulating precursor cells exhibited a strong CD34 signal on day 1 in culture; however, 30% of them displayed CD34 staining on day 21 in culture. By contrast, circulating precursor cells did not express involucrin on day 1, but they exhibited strong involucrin signal on day 21 in our established culture medium. Scale bars: 20 μ m (**A**); 100 μ m (**B**).

stable throughout time to track their epithelial transition process.

Circulating Precursor Cells Transdifferentiate into Epithelial-Like Cells

To demonstrate further the role of circulating precursor cells in PBMC transdifferentiation into epithelial-like cells, we isolated circulating precursor cells from whole blood and then resuspended them under similar culture condition. The morphology of the pure progenitor cell population was similar to that of total PBMCs when they were in our established culture medium. Thus, after 21 days in culture, they exhibited larger sizes and polygonal shapes. Similarly, these cells had the capacity to transdifferentiate into epithelial-like cells and became positive for 14-3-3 σ and keratin-5, -8 on day 21 in culture (data not shown). In addition, as shown by confocal microscopy (Figure 4A), these epithelial-like cells exhibited an organized cytokeratin network spreading through the whole cytoplasm. This pattern was more homogeneous and denser than that in keratinocytes used as a positive control. Interestingly, in both cell types, the filaments of cytokeratin co-localized with 14-3-3 σ . As expected, circulating precursor cells displayed a strong CD34 staining on day 1 in culture, which was fading down during the course of cell transdifferentiation process into epithelial-like cells (Figure 3B). Thus, 30% of these transdifferentiated precursor cells were positive for CD34 staining on day 21 in culture. On the other hand, the involucrin was negative on day 1 and became positive on day 21 in culture, and this finding was consistent with the epithelial-like pattern seen in these cells (Figure 4B).

Epithelial-Like Cells Express and Release 14-3-3 σ into the Conditioned Media

As noted in the Introduction, this protein is not only a long-lasting marker to trace epithelial differentiation, but it also has the potential to induce functional profile modification on other cells when released into the surrounding environment. Thus, to identify and quantify the release of 14-3-3 σ into conditioned media by PBMC-derived epithelial-like cells, an ELISA was performed. This experiment was conducted using three sets of weekly collected PBMC-conditioned media. According to the Kruskal-Wallis test, the variation among medians of 14-3-3 σ concentration at different time points of epithelial-like transdifferentiation is very significant with a P value <0.01 . As shown in Figure 5, the release of 14-3-3 σ was markedly higher in conditioned media collected from PBMCs at days 28 and 35 (range, 0.81 to 2.79 pg/ μ l) compared with those collected from PBMCs at days 0, 7, 14, 21, 42, and 49.

PBMC-Derived Epithelial-Like Cells Stimulate the MMP-1 Expression in Dermal Fibroblasts

To demonstrate the effect of releasable 14-3-3 σ from PBMC-derived epithelial-like cells on dermal fibroblast

Figure 5. 14-3-3 σ release into the conditioned media by PBMC-derived epithelial-like cells. Using ELISA, the content of 14-3-3 σ released by PBMC-derived epithelial-like cells into the conditioned media was measured at different time points of cell transdifferentiation (days 0, 7, 14, 21, 28, 35, 42, and 49). The 14-3-3 σ concentrations are expressed in picograms per microliter. The multiple sample group's analysis (Kruskal-Wallis test) revealed a very significant variation with a P value <0.01 .

MMP-1 expression, a co-culture system was established. The former cells were cultured on inserts (upper chambers) and placed in six-well plates with subconfluent dermal fibroblasts (bottom chambers) for 24 hours at 37°C. Because of their functional nature and role in both normal and pathological wound healing, MMP-1 expression was selected as a functional assay to study the dermal fibroblast response to soluble products released by PBMCs at different time points of epithelial-like cell differentiation. Thus, the Western blot analysis demonstrated that MMP-1 expression was significantly higher when fibroblasts were exposed to undifferentiated PBMCs at day 0 in culture compared with negative control F/F (fibroblasts co-cultured with fibroblasts) with a P value of 0.01 (Figure 6). This initial overexpression decreased when fibroblasts were co-cultured with either PBMCs on day 7, day 14, or day 21. When compared with negative control (F/F), these three time points did not achieve statistical significances (Figure 6B). However, MMP-1 expression increased again when fibroblasts were co-cultured with PBMCs in advanced state of epithelial-like differentiation. Specifically, MMP-1 overexpression was observed in fibroblasts receiving conditioned media collected from PBMCs on day 28 (P value, 0.01), day 35 (P value, 0.0079), and day 42 (P value, 0.0079) (Figure 6B). However, although a marked increase in MMP-1 expression in fibroblasts co-cultured with PBMCs on day 49 was observed, this increase was not significant because of high variability.

14-3-3 σ Released by PBMC-Derived Epithelial-Like Cells Stimulates the MMP-1 Expression in Dermal Fibroblasts

To identify which stimulating factors are involved in the increase of MMP-1 expression in dermal fibroblasts by PBMC-derived epithelial-like cells, we first examined the expression of tumor necrosis factor- α and interleukin-1. We were unable to detect these cytokines in PBMC-derived epithelial-like cell-conditioned media using ELISA (data not shown). Therefore, we started to

Figure 6. MMP-1 expression in dermal fibroblasts co-cultured with PBMCs. Western blot and densitometric analysis of MMP-1 expression in fibroblasts co-cultured with PBMCs at different time points. **A:** Western blot shows the expression of MMP-1 in fibroblasts co-cultured with PBMCs at days 0, 7, 14, 21, 28, 35, 42, and 49 in established culture conditions. **Lane F/F** corresponds to negative control (fibroblasts co-cultured with fibroblasts) and **lane F/K** to positive control (fibroblasts co-cultured with differentiated keratinocytes). **B:** In densitometric analysis, values are expressed as MMP-1/β-actin ratio. Compared with negative control F/F, MMP-1 expression is significantly higher when fibroblasts are exposed to PBMCs from overnight incubation (**P* value, 0.01). Then, MMP-1 reduces its expression when fibroblasts are exposed to PBMCs on days 7, 14, or 21. These three time points did not present statistical significance compared with control F/F. The MMP-1 expression increased again when fibroblasts were co-cultured with advanced differentiated PBMCs of day 28 (***P* value, 0.01), day 35 (***P* value, 0.0079), and day 42 (*****P* value, 0.0079). The MMP-1 expression in dermal fibroblasts exposed to PBMCs on day 49 showed high variability. Data were calculated from three independent experiments.

focus on evaluating the 14-3-3σ taking into account that this protein is expressed in PBMC-derived epithelial-like cells after day 7 in culture (Figure 1) and then released into their conditioned media (Figure 5). Our group already described a soluble fraction released by differentiated keratinocytes.³⁵ Thus, we decided to treat subconfluent cultures of dermal fibroblasts with PBMC-conditioned media from day 28 of epithelial-like differentiation because of their capacity of inducing cell proliferation (data not shown) and MMP-1 expression in dermal fibroblasts.

To examine directly the role of 14-3-3σ released by PBMC-derived epithelial-like cells on MMP-1 expression in dermal fibroblasts, we conducted a 14-3-3σ immunoprecipitation (IP). With this method, the expression of MMP-1 by dermal fibroblasts after treatment with 14-3-3σ-depleted PBMC-conditioned media was partially prevented when compared with full-content PBMC-conditioned media. As shown by Western blotting (Figure 7A), the 14-3-3σ IP reduced MMP-1 expression in dermal fibroblasts after treatment with either PBMC-conditioned media collected on day 28 or recombinant 14-3-3σ. In addition, the densitometric analysis of three independent experiments expressed as MMP-1/β-actin ratio showed that the removal of 14-3-3σ from PBMC-conditioned media reduced the expression of MMP-1 by 39.5% when

Figure 7. PBMC-derived conditioned media immunoprecipitation: The effect on MMP-1 expression in dermal fibroblasts. **A:** MMP-1 expression on dermal fibroblasts after treatment with either full-content PBMC-conditioned media collected on day 28 (IP-) or supernatants derived from a 14-3-3σ immunoprecipitation (IP+). As negative control, dermal fibroblasts treated with DMEM (49%) and KSFM (49%), plus 2% FBS were used. The positive control corresponds to dermal fibroblasts treated with 14-3-3σ at 4 μg/ml (IP-) or after 14-3-3σ depletion with immunoprecipitation (IP+). **B:** Densitometric analysis is expressed as MMP-1/β-actin ratio. Data are expressed as mean ± SD from three independent experiments. **P* < 0.05.

compared with full-content PBMC-conditioned media (Figure 7B). This result attained statistical significance with a *P* value of 0.05 (Figure 7B).

Discussion

To establish cell transdifferentiation, the identification of switch on/off phenomena in expression of certain specific markers under minimal cell manipulation is necessary.³⁶ Thus, after testing several culture medium cocktails with different proportions of media formulated to either fibroblast or keratinocyte cultures, the combination containing 49% DMEM, 49% defined KSFM, and defined keratinocyte-SFM growth supplement plus 2% FBS appeared to be adequate as an inducing factor to support the PBMC transdifferentiation into epithelial-like cells and cell survival for more than 50 days. Under this experimental condition with minor intervention of external factors, a subset of PBMCs can survive and be maintained in culture by modifying their size, morphology, and protein expression in a time-dependent manner. These cells exhibit an initial round morphology with 6- to 10-μm diameter that change transiently into either spindle-shaped or stellate cells at day 7 to finally become polygonal cells with 80- to 120-μm size at day 21 in culture (Figures 1 and 3). In addition, when the cell number is appropriate, these cells contact each other to occupy all spaces of the culture plate surface and become similar to those of

confluent epithelial cells in cultures. However, it is not clear whether these epithelial-like cells have the capacity to produce desmosomal components and develop multilayer structure similar to those of epidermal layers. Thus, a systematic study is needed not only to address this question but also to explore and compare other issues relevant to biological function of these cells in *in vitro* and *in vivo* models.

Although the circulating precursor cells have the capacity to transdifferentiate into epithelial-like cells when they are in our established culture condition, it remains to be seen whether other cell subsets within PBMCs may follow a similar differentiation process into epithelial-like cells. For instance, monocytes have been described as a source of fibrocytes, a fibroblast-like cell type that participates in some clinical conditions and in tissue repair.^{22,27,37} Changes in the cell commitment of both PBMCs and circulating precursor cells can be tracked using specific markers for epithelial differentiation. As PBMCs, circulating precursor cells developed a cell transdifferentiation into epithelial-like cells characterized by cytokeratin⁺, involucrin⁺, CD34^{lo}, and 14-3-3 σ ⁺ profile.

Cytokeratins are polypeptides that constitute the most abundant type of intermediate filaments in epithelial cells.^{38,39} Thus, the expression of several cytokeratins in both PBMCs and circulating precursor cells in treatment with our established culture medium allowed us in this study to confirm their epithelial determination. In support of this, we also showed that involucrin, which is a component of mature epithelial cells,^{40,41} is also expressed by epithelial-like cells in our culture condition used.

It is interesting to observe that the glycoprotein CD34 is either markedly reduced or disappeared in circulating precursor cells during the course of the transdifferentiation process of PBMCs into epithelial-like cells. In fact, only 30% of the purified CD34⁺ precursor cell population expressed very low levels of this marker on differentiation on day 21 in culture. Even though it is expressed by multipotent hematopoietic progenitors, CD34 is lost during the differentiation process and disappears on mature hematopoietic cells.⁴² There is evidence suggesting that CD34 plays a critical role in maintaining hematopoietic cells in an immature stage by preventing their terminal differentiation.^{43,44} Thus, a reducing trend of CD34 expression seen here might also be related to the potential role of CD34 family of sialomucins in regulating cell adhesion. Thus, the highly glycosylated CD34 allows the homing of stem cells to the bone marrow compartment.^{45,46} However, the CD34 variant present on activated hematopoietic stem cells (as circulating precursor cells) may help in their migration and recruitment to other microenvironments.^{47–49} In our study, we showed that circulating precursor cells have the capacity to become epithelial-like cells by reducing the expression of CD34 and gaining the presence of epithelial markers such as 14-3-3 σ and keratin proteins.

From markers used in this study, 14-3-3 σ requires special attention. As is widely documented, despite being ubiquitously expressed within cells, 14-3-3 proteins interact with more than 200 molecules and exert numerous crucial intracellular functions such as control of cell

cycle and apoptosis, regulation of signal transduction pathways, cell proliferation and differentiation, cell survival, cell fate, cellular trafficking, and protein folding and processing, among others.^{50–55} In addition, 14-3-3 proteins bind to keratin in epithelial cell intermediate filaments modulating their structural organization and cell mitotic progression.^{56,57} In this regard, 14-3-3 σ may play a role in events associated with morphological changes that lead to epithelial-like commitment of both PBMCs and circulating precursor cells. In our study, the expression of 14-3-3 σ was detected after 7 days in culture while cells progressed into a morphological and functional epithelial-like determination (Figures 1, 2, and 3). Based on confocal microscopy, 14-3-3 σ was co-localized with filaments of cytokeratins in cytoplasm of epithelial-like cells, although it displays a different pattern compared with keratinocytes used as a positive control. Therefore, as the cytoskeleton provides mechanical support to many cell functions, the appearance of these proteins confirm the transdifferentiation of these cells into epithelial-like cells, which probably have a different biological function that needs to be elucidated. However, similar to keratinocytes,⁵³ these transdifferentiated epithelial-like cells released the 14-3-3 σ into the conditioned media, and subsequently modulated the MMP-1 expression in dermal fibroblasts in a time-dependent manner (Figures 5, 6, and 7, respectively). Thus, using a co-culture system, the PBMCs induced a bimodal curve of MMP-1 overexpression in dermal fibroblasts with both hyperacute and late peaks (Figure 6). In this study, we were unable to demonstrate any relationship between the MMP-1 response and interleukin-1 and tumor necrosis factor- α overexpression. Therefore, we consider the hyperacute peak as a brief response most likely to be attributable to either other stimulating factors or a nonphysiological release of inflammatory mediators after PBMC isolation from whole blood. For that reason, we focus our attention on the late peak of MMP-1 and the potential role of 14-3-3 σ in that fibroblast's response. Unlike the intracellular functions, the existence of 14-3-3 protein soluble forms and their biological effects on surrounding cells are relatively unknown. Ghahary and colleagues^{33,35} provided the initial documentation about the capacity of differentiated keratinocytes to release 14-3-3 σ into keratinocyte-conditioned medium (KCM). In this study, the MMP-1 expression peaked when dermal fibroblasts received conditioned media from PBMCs on day 28 and remained high up to day 42. This period exhibited statistical significance when compared with negative control. Although a marked increase in expression of MMP-1 in cells receiving PBMC-conditioned media on days 7, 14, 21, and 49 was observed relative to untreated control, the difference was not significant because of a large variation in responses of different fibroblast cell strains used. This variation may also have been generated during the long cell incubation needed for PBMCs to transdifferentiate into epithelial-like cells. Although there is a need for much work to be performed in this field, the discovery of 14-3-3 σ as both long-lasting marker for PBMC/stem cell differentiation into epithelial-like cells and having MMP-1-modulating effect on dermal fibroblasts has major implications in tissue repair process. It is interesting to ob-

serve that a releasable form of 14-3-3 σ from PBMC-derived epithelial-like cells can regulate the MMP-1 expression in dermal fibroblasts. This biological effect might be attributable to an increase in MMP-1 promoter activity by p38 mitogen-activated protein kinase (MAPK) as occurred in differentiated keratinocytes.⁵⁸ The apparent discrepancy between the beginning of 14-3-3 σ expression in PBMC-derived epithelial-like cells (day 7 in culture) and the time point at which these cells induce the late MMP-1 peak in dermal fibroblasts in co-culture system (day 28 in culture) may be explained by maturation of the cytoplasmic secretory machinery to successfully release the extracellular fraction of the protein into the conditioned media.

Findings from this study provide new insights into the potential role of PBMCs and circulating precursor cells to generate epithelial-like cells. The understanding of this particular cell transdifferentiation would facilitate the treatment of chronic nonhealing wounds (seen in elderly, diabetic, and immunocompromised patients) as well as those associated with overhealing wounds such as postburn hypertrophic scarring. Thus, the identification of a releasable form of 14-3-3 σ in PBMC-derived epithelial-like cells reveals new alternatives to control the fibrogenic process associated with tissue injury. In this regard, it is not unreasonable to postulate that bone marrow-derived cells may migrate to the injured area and transdifferentiate into transitional cells that develop either fibrogenic or anti-fibrogenic profile according to the repertoire of cytokines and stimulating factors present in the wound milieu.⁴⁶ Interestingly, this precept raises the possibility that the balance of biochemical signaling at the local level may be very critical to define the cell differentiation pathway. Thus, once these bone marrow-derived cells engraft injured tissues and acquire one of the transformed profiles, they may cross talk with dermal fibroblasts, controlling their behavior and subsequently modulating the extracellular matrix production, with the final result of wound healing. In support of this unexpected and multilineage differentiation of adult stem cells, when PBMCs are exposed to conditions formulated for fibroblast culture, they become fibrocytes, a type of terminally differentiated cells that increase the collagen production by themselves and by stimulating surrounding fibroblasts.^{22,26,27,37,59}

Like any other novel finding, many questions have been raised here that can be addressed by further studies: i) What is the source of these cells and their percentage in circulation? ii) What is the impact of these cells in wound healing? iii) Can the local environment induce these specific somatic cell determinations? iv) Can they form multilayers and grow on matrix? v) How do they control the fibroblast behavior? In addition, extrapolations to clinical conditions include the following: vi) How could the blood supply quality at an injured area influence this transitional epithelial-like cell model? vii) What could be the role of local infection in the somatic determination of these cells within the system? viii) What is the impact of the ethnic background in the cell behavior?

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