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Peripheral Blood Fibrocytes: Differentiation Pathway and Migration to Wound Sites[1]

Riichiro Abe,* Seamas C. Donnelly,* Tina Peng,* Richard Bucala,† and Christine N. Metz[2]*

Fibrocytes are a distinct population of blood-borne cells that display a unique cell surface phenotype (collagen I^+/$CD11b^+$/$CD13^+$/$CD34^+$/$CD45RO^+$/MHC class II^+/$CD86^+$) and exhibit potent immunostimulatory activities. Circulating fibrocytes rapidly enter sites of tissue injury, suggesting an important role for these cells in wound repair. However, the regulatory processes that govern the differentiation of blood-borne fibrocytes and the mechanisms that underlie the migration of these cells to wound sites are currently not known. We report herein that ex vivo cultured fibrocytes can differentiate from a $CD14^+$-enriched mononuclear cell population and that this process requires contact with T cells. Furthermore, we demonstrate that TGF-β1 (1–10 ng/ml), an important fibrogenic and growth-regulating cytokine involved in wound healing, increases the differentiation and functional activity of cultured fibrocytes. Because fibrocytes home to sites of tissue injury, we examined the role of chemokine/chemokine receptor interactions in fibrocyte trafficking. We show that secondary lymphoid chemokine, a ligand of the CCR7 chemokine receptor, acts as a potent stimulus for fibrocyte chemotaxis in vitro and for the homing of injected fibrocytes to sites of cutaneous tissue injury in vivo. Finally, we demonstrate that differentiated, cultured fibrocytes express α smooth muscle actin and contract collagen gels in vitro, two characteristic features of wound-healing myofibroblasts. These data provide important insight into the control of fibrocyte differentiation and trafficking during tissue repair and significantly expand their potential role during wound healing. *The Journal of Immunology,* **2001, 166: 7556–7562.**

Fibroblasts, depending on their tissue source and stimuli for activation, are a heterogeneous population of cell types exhibiting distinct functions. Fibroblasts found in the wound are considered essential for the healing process. The concept that wound fibroblasts can originate from peripheral blood cells goes back almost 100 years (reviewed in Ref. 1). Since then, numerous studies have reported the differentiation of peripheral mononuclear cells into fibroblast-like cells.

In 1994, a distinct population of blood-borne fibroblast-like cells that rapidly enter sites of tissue injury was described (2). Termed fibrocytes, these cells comprise 0.1–0.5% of nonerythrocytic cells in peripheral blood and display an adherent, spindle-shaped morphology when cultured in vitro. Cultured fibrocytes express the fibroblast products collagen (Col)[3] I, Col III, and fibronectin, as well as the leukocyte common Ag (CD45RO), the pan-myeloid Ag (CD13), and the hemopoietic stem cell Ag (CD34). In addition, fibrocytes express MHC class II and costimulatory molecules (CD80 and CD86) and have the capacity to present Ag in vitro and in vivo (3, 4). By their morphology, growth properties, and cell surface markers, fibrocytes appear to be distinct from monocytes/macrophages, dendritic cells, and other known APC types. Cultured fibrocytes do not express typical monocyte/macrophage-specific or B cell markers (such as CD14, CD16, or CD19), nor do they express typical surface proteins of dendritic cells or their precursors (such as CD1a, CD10, CD25, and CD38). In addition, fibrocytes isolated from peripheral blood and cultured ex vivo secrete a unique profile of cytokines, growth factors, and chemokines (5).

Based on their presence in wounds and their secretion of proinflammatory cytokines, chemokines, and extracellular matrix proteins, fibrocytes have been postulated to play a role in wound healing and connective tissue formation. Although initial studies performed in sex-mismatched bone marrow chimeric mice suggested that fibrocytes arose from a relatively radioresistant progenitor population (2), the precise origin of these cells and the wound trafficking signals relevant to their directed migration remain unknown. In this study we identify a differentiation pathway of cultured fibrocytes, characterize the signals for fibrocyte migration to wound sites in vivo, and reveal the potential role of fibrocytes in wound contracture.

Materials and Methods

Mice

BALB/c mice (females, 8–12 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal procedures were conducted according to guidelines of the institutional animal care and use committee of North Shore University Hospital under an approved protocol.

Antibodies, cytokines, and chemokines

FITC-anti-α smooth muscle actin (FITC-anti-αSMA) mAb was purchased from Sigma (St. Louis, MO). Biotinylated rabbit anti-Col I and biotinylated rabbit IgG were purchased from Rockland (Gilbertsville, PA). Anti-mouse CCR3, CCR5, CCR7, or CXCR4 polyclonal Abs and FITC-anti-goat IgG Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other Abs were purchased from BD PharMingen (San Diego, CA). TGF-β1 (active), secondary lymphoid chemokine (SLC), and stromal-derived cell factor (SDF) were purchased from R&D Systems (Minneapolis, MN).

Cells

Fibrocytes (human and mouse) were purified from peripheral blood and cultured as previously described (2, 5). Briefly, PBMCs were isolated from

Laboratory of *Vascular Biology and †Medical Biochemistry, The Picower Institute for Medical Research, Manhasset, NY 11030

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[2] Address correspondence and reprint requests to Dr. Christine N. Metz, The Picower Institute, 350 Community Drive, Manhasset, NY 11030. E-mail address: cmetz@picower.edu

[3] Abbreviations used in this paper: Col, collagen; SLC, secondary lymphoid chemokine; αSMA, α smooth muscle actin; SDF, stromal-derived cell factor.

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human Leukopaks (purchased from the Long Island Blood Center, Long Island, NY) by centrifugation over Ficoll/Paque (Pharmacia, Piscataway, NJ) following the manufacturer's protocol. After 2 days of culture on tissue culture flasks in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 20% FBS (HyClone Laboratories, Logan, UT), penicillin, streptomycin, and L-glutamine, nonadherent cells were removed by gentle aspiration, and media were replaced. After 10–12 days, adherent cells were lifted by incubation in ice-cold 0.05% EDTA (in PBS). The crude fibrocyte preparations (~70–80% pure based on Col I/CD11b staining) then were depleted by immunomagnetic selection of contaminating T cells (~13%), B cells (~3%), and monocytes (~11%) using pan-T, anti-CD2; pan-B, anti-CD19; and anti-CD14 Dynabeads, respectively (Dynal, Great Neck, NY). The resultant cultured, enriched fibrocyte populations were ≥95% pure based on Col I/CD11b staining, with T cells and monocytes contributing ~3 and 2%, respectively. Typically, between 0.4 and 5×10^4 fibrocytes were isolated per milliliter of human blood.

Mouse PBMC were isolated from BALB/c mouse blood (heparinized) obtained by cardiac puncture following CO_2 asphyxiation. Mouse blood was mixed with PBS (2:1) and layered over Ficoll/Paque (Pharmacia; 15 ml blood over 30 ml Ficoll) and centrifuged according to the manufacturer's protocol. Mouse fibrocytes were cultured in DMEM supplemented with 10% FBS and 10% mouse serum (Sigma), penicillin, streptomycin, and L-glutamine, as previously described (4). After 10–12 days, the adherent crude fibrocyte preparation (~75% pure based on Col I/CD11b staining) were lifted using 0.05% EDTA in PBS and depleted by immunomagnetic selection of contaminating T cells, B cells, and monocytes using pan-T (anti-CD90), pan-B (anti-B220) Dynabeads (Dynal), and anti-mouse CD14 attached to Dynabeads, respectively. Following immunodepletion, the cultured, enriched fibrocyte preparations were verified to be ≥95% pure by Col I^+/$CD11b^+$ staining as determined by flow cytometry. Approximately $0.8–4 \times 10^4$ fibrocytes/ml mouse blood (~1–1.2 ml blood/mouse) were purified.

Human adult dermal fibroblasts were purchased from Clonetics (San Diego, CA) and cultured according to the manufacturer's recommendations. The human intestinal smooth muscle cell line was obtained from American Type Culture Collection (Manassas, VA) and cultivated according to recommended procedures.

Analysis of fibrocyte differentiation

Initial studies were aimed toward elucidating the cellular origin of peripheral blood fibrocytes. Therefore, we fractionated whole blood supplied as Leukopaks (see Fig. 1*A*) and cultured the various fractions in vitro. Adherent cells were collected from overnight cultures of human PBMCs (total), and $CD14^+$ cells were enriched from the PBMC fraction by depletion of T and B cells ($CD14^+$). $CD14^-$ cells (including all PBMCs except $CD14^+$ cells) were purified by depletion of the $CD14^+$ cells from the total PBMC preparation. Using the Transwell two-chamber system (0.4 μm; Corning Costar, Cambridge, MA), $CD14^+$, $CD14^-$, or total cells (3×10^6 cells/ml in DMEM/10% FBS) were cultured in either the upper or lower chambers, as indicated. After 7 days of culture, the cells that grew in the lower well were collected and analyzed for fibrocyte-like differentiation by Col I/CD11b staining and flow cytometry. Similar results were observed with cells prepared from three other donors.

For studies investigating a requirement for T cells in fibrocyte differentiation, the $CD14^+$ cell fraction (see above) was purified from purchased Leukopaks and cultured with autologous T cells isolated using T cell enrichment columns (R&D Systems). T cell purity was ≥95%, as assessed by flow cytometry using anti-CD3 Abs (PharMingen). After 7 days of coculture, the resulting population was analyzed for the percentage of fibrocytes by Col I/CD11b staining and flow cytometry. Similar results were observed using fibrocytes isolated from three different donors.

Flow cytometric analysis

For single Ab staining, cells (10^5 aliquots) were resuspended in PBS containing 3% BSA and 0.1% sodium azide (FACS buffer) and incubated with the indicated Abs (or labeled isotype control Abs) for 30 min at 4°C. In cases where the primary Abs were not labeled, cells were washed and incubated with revealing Abs diluted in FACS buffer. After washing the cells in FACS buffer, fluorescence data were acquired on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed using CellQuest software (BD Biosciences). At least 5000 cells were analyzed per condition. To analyze preparations for Col I/CD11b staining, cells were prepared as described above and first incubated in FACS buffer containing biotinylated Col I Ab (or biotinylated rabbit control IgG), then washed and incubated sequentially in FACS buffer containing FITC-streptavidin (PharMingen) and PE-CD11b (PharMingen). Intracellular staining for αSMA was performed as previously described (6, 7). Briefly, cells were fixed and permeabilized using the Perm/Fix kit (PharMingen) according to the manufacturer's recommendations and incubated with FITC-anti-αSMA mAb (Sigma).

Fibrocyte migration in vivo using a wound model

Cultured, enriched peripheral blood-derived mouse fibrocytes (>96% pure) were stained with a membrane-inserting red dye, PKH-26 (Sigma), following the manufacturer's protocol. Labeling efficiency, assessed by flow cytometry, and viability, assessed by trypan blue exclusion were >85%. PKH-labeled cells (5×10^5) in 100 μl PBS were administered into the tail vein (i.v.) of BALB/c mice ($n = 2$/group). Immediately following injection of the enriched fibrocytes, a full-thickness round skin wound (5-mm diameter) was made in the dorsal subscapular area of each recipient mouse by excision with skin punch equipment, as previously described (8). Wound sites were removed 4 days later and examined for the presence of fluorescent fibrocyte cells by microscopic analysis of thin frozen sections and by quantitative flow cytometric analysis following proteolytic digestion. For quantitative flow cytometric analysis, excised skin (250 μg biopsy/animal) was chopped into small fragments, then incubated for 1 h at 37°C in RPMI 1640 containing 10% FBS, 2 mg/ml collagenase, and 20 μg/ml DNase I. The resulting single-cell suspension was examined by flow cytometry to determine the number of fluorescent fibrocytes present using calibration beads as previously described (9).

RT-PCR

Total RNA was isolated from cultured, enriched fibrocytes (>95% pure) using RNAzol B (Tel-Test, Friendswood, TX). The cDNA was prepared from 1.0 μg RNA using 0.25 ng oligo-$(dT)_{12–18}$ and Moloney murine leukemia virus reverse transcriptase following the protocol supplied by the manufacturer protocol (Life Technologies). Two-microliter aliquots of cDNA were amplified by PCR using Supermix (Life Technologies) in a Perkin-Elmer model 9600 thermal cycler using specific primers PCR pairs as previously described: αSMA (10); CCR3 (11); CCR4, CCR5, and CXCR3 (12); CCR6 (13); CCR7 (14); CXCR4 (15); and β-actin (sense primer, 5′-GTGGGGCGCCCCAGGCACCA-3′; antisense primer, 5′-CTCCTTAATGTCACGCACGATTTC-3′). Thermal cycling (25–30 cycles, in 25 μl) was performed as follows: denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min, and extension at 72°C for 1 min. PCR products were separated by electrophoresis through 2% agarose gels and viewed under UV light after ethidium bromide staining. To control for potential genomic DNA contamination, PCR were performed without the reverse transcription step, and no DNA amplification products were detected.

In vitro fibrocyte chemotaxis assay

Chemotaxis assays were performed using Costar Transwell inserts (8-μm pore size) according to the manufacturer's protocol. Cultured, enriched fibrocytes (≥95% pure) were resuspended at 1×10^6 cells/ml in DMEM containing 0.1% BSA. Medium alone (negative control) or medium containing SLC or SDF (600 μl) was added to individual wells of a 24-well plate. Transwell devices then were inserted, and the fibrocytes (100 μl) were layered on top of the membrane ($n = 3$ wells/condition). After 3 h the transmigrated cells were collected and counted by flow cytometry using calibration beads (Coulter, Miami, FL), as previously described (9). Similar results were observed with two additional donors. For checkerboard analysis of SLC-directed chemotaxis of fibrocytes, 100 ng/ml SLC was added to either the top or bottom chamber alone and to both the bottom and top chambers (see Fig. 5*B*).

In vivo fibrocyte chemotaxis assay

Immediately following tail vein injection of PKH-labeled enriched fibrocytes (>94% pure; 5×10^5 cells/mouse), BALC/c mice received an i.d. injection of SLC, SDF (0.1 or 1 μg in 50 μl), or PBS alone in the scapular region of the back (shaved). The injected site was excised 4 h later and proteolytically digested to produce a single-cell suspension (as described above). The number of labeled fibrocytes per biopsy sample (250 μg) was estimated by flow cytometry using calibration beads (9). This experiment was repeated twice with similar results.

Collagen lattice contraction assay

Cellular collagen gel contraction assays were performed as previously described (16). Overnight adherent PBMC cultures, 10-day-old enriched fibrocytes (≥95% pure) previously cultured in the absence or the presence of TGF-β1 (10 ng/ml for 7 days before experiment), or normal human dermal fibroblasts were lifted using cold EDTA/PBS solution. A collagen

solution in DMEM was prepared from rat tail Col I according to the manufacturer's instructions, and combined with cells at 2×10^5/ml ($n = 3$/cell type). The collagen/cell mixture (400 μl/well) was dispensed into culture plates and allowed to polymerize at 37°C for 30 min. Immediately after polymerization, 2 ml DMEM containing 10% FBS was added to each well. The gels then were detached from the wells by gently shaking the culture plates at various time points (0, 24, 48, and 72 h), and the longest and shortest diameters of each gel were measured. The mean of the linear measurements ($n = 3$ for each sample) taken at each time point was used to estimate the contractility of the cells. The data are presented as percent gel contraction. This experiment was repeated twice with similar results using cells obtained from different donors.

Results

A peripheral blood population consisting predominantly of CD14$^+$ cells, but not a CD14$^-$ cell population, gives rise to fibrocytes in vitro

To determine the origin of fibrocytes, we analyzed the growth and phenotype of adherent human PBMC cultured on plastic (Fig. 1*A*). After standard Ficoll separation, the resulting population was ~40–50% CD14$^+$ cells. Following an overnight adherence step, the adherent cell population (total) was >70% CD14$^+$ cells exhibiting no detectable Col I staining, as assessed by flow cytometry (data not shown) (5). We have shown in previous studies that after 2 wk cells in these cultures no longer express CD14, but do express Col I (5). Importantly, we found that a cell population enriched for CD14$^+$ cells, (i.e., PBMCs depleted of all T or B cells by magnetic beads) gives rise to very few Col I$^+$/CD11b$^+$ spindle-shaped fibrocytes after 1 wk of culture (data not shown).

Using Transwell culture chambers, we examined the cellular requirements for fibrocyte differentiation (CD11b/Col I$^+$) in vitro from circulating blood cell fractions (Fig. 1*B*). When a CD14$^-$ cell fraction was cultured in the lower well of a Transwell plate and total PBMCs were cultured in the top chamber for 1 wk, no fibrocytes appeared in the lower chamber. Similarly, no fibrocytes appeared in the lower chamber when CD14$^+$ cells alone were cultured in the bottom chamber and CD14$^+$ cells or total PBMCs were cultured in the top chamber for 1 wk. By contrast, when total PBMCs were cultured in the bottom well of the Transwell chamber and either CD14$^-$ cells or CD14$^+$ cells (or medium alone, data not shown) were cultured in the top chamber, numerous spindle-shaped fibrocytes (CD11b$^+$/Col I$^+$) were observed within 1 wk. These data suggest that fibrocyte outgrowth from cultured PBMCs requires cellular interaction between a population of enriched CD14$^+$ cells and another peripheral blood cell type or that fibrocyte precursors are only present in the PBMC fraction.

To examine the requirement of cellular interaction, we then added either purified, autologous T or B cells to CD14$^+$ cell cultures in various ratios (CD14$^+$:T, 0:1, 1:0, 3:1, 1:1, and 1:3) for 7–10 days and found that cocultures of CD14$^+$ cells and T cells give rise to fibrocytes (CD11b$^+$/Col I$^+$; Fig. 1*C*). We observed that a CD14$^+$cell:T cell ratio of 3:1 was optimal (Fig. 1*C*) for culturing fibrocytes. By contrast, no fibrocytes appeared when T cells were cultured alone or in cocultures of B and CD14$^+$ cells or when CD14$^+$ cells were cultured with T cell-conditioned medium (data not shown). Because fibrocytes do not express T cell markers (CD2, CD3, CD4, and CD8) or typical T cell cytokines (IL-2, IL-4, and IFN-γ), it is unlikely that T cells give rise to fibrocytes.

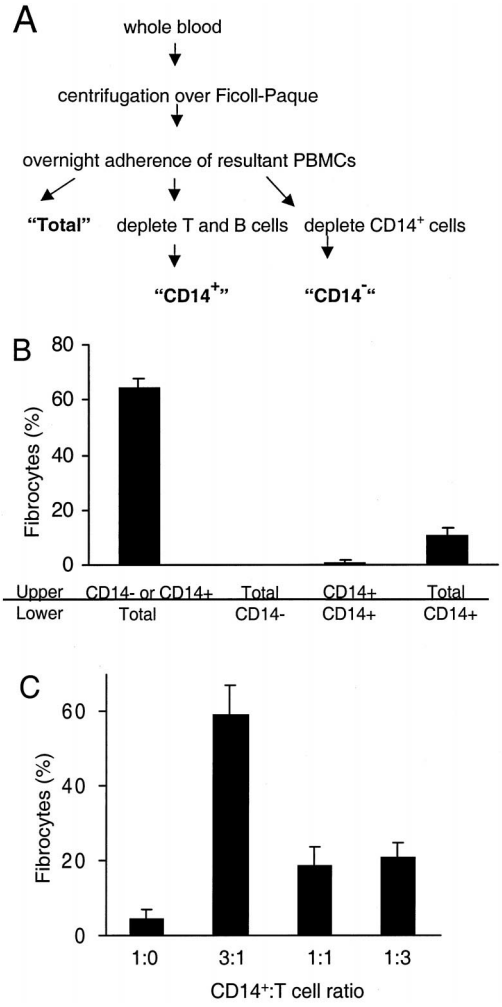

FIGURE 1. Fibrocytes differentiate in vitro from a blood-derived CD14$^+$ population and require direct T cell interaction. *A*, Schematic representation of the experimental design. Adherent cells from a human PBMC fraction isolated from whole blood were collected after an overnight incubation (designated Total). A CD14$^+$ enriched population was isolated from the Total fraction by depletion of T and B cells (designated CD14$^+$), and a CD14$^-$ enriched population was isolated from the Total fraction by depletion of CD14$^+$ monocytes (designated CD14$^-$). *B*, Using the Transwell culture system (0.4 μm), total adherent PBMCs (Total), CD14$^+$-enriched cells or total PBMCs depleted of T and B cells (CD14$^+$), and total PBMCs depleted of CD14$^+$ cells (CD14$^-$) were cultured in the upper and lower chambers, as indicated, for 7 days. Cells in the lower chambers were lifted and analyzed for fibrocyte phenotype using CD11b$^+$/Col I$^+$ staining by flow cytometry, and data are represented as the percentage of fibrocytes. *C*, CD14$^+$ enriched cells (PBMCs depleted of both T and B cells) were incubated with various ratios of autologous T cells, and the resulting crude fibrocyte cultures were analyzed for fibrocyte markers after 7 days in culture by flow cytometry. Data show the percentage of fibrocytes based on CD11b$^+$/Col I$^+$ staining.

TGF-β1 accelerates fibrocyte differentiation in vitro

Next, we examined whether TGF-β1, a cytokine important for fibroblast proliferation and extracellular matrix production could promote the differentiation and accumulation of fibrocytes within PBMC cultures. The addition of TGF-β1 (1–10 ng/ml) to PBMC cultures on days 3–10 promoted fibrocyte differentiation in vitro, as shown by the enhanced accumulation of cells with spindle-shaped morphology (Fig. 2, *A–C*). Treatment of these cultures with TGF-β1 increased the expression of Col I by fibrocytes within these cultures in a dose-dependent manner (Fig. 2*D*). The mean fluorescence intensities for Col I expression were 11, 24, and 63 for fibrocytes in cultures treated with 0, 1, and 10 ng/ml TGF-β1, respectively (Fig. 2*D*). These Col I$^+$ cells also stained positively for CD11b (data not shown). Furthermore, there was a dose-dependent increase in the number of fibrocytes within the cultures that stained positively for Col I in response to TGF-β1, with almost a 40% increase in response to 10 ng/ml TGF-β1 compared

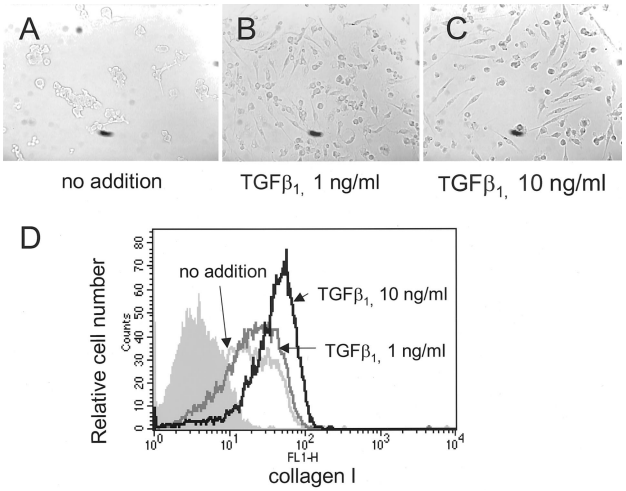

FIGURE 2. TGF-β1 promotes the differentiation of fibrocytes. Four days following their isolation from human blood, crude fibrocyte cultures were treated with various concentrations of TGF-β1. Then 7 days later cultures were examined for spindle-shaped morphology. Representative cultures photographed at $\times$200 are shown: *A*, no addition; *B*, 1 ng/ml TGF-β1; and *C*, 10 ng/ml TGF-β1. *D*, TGF-β1-treated (0–10 ng/ml) crude fibrocyte cultures were lifted, stained for Col I, and analyzed by flow cytometry. Isotype control staining of cells is shown as a shaded histogram. The *y*-axis represents relative cell number, and the *x*-axis represents mean fluorescence intensity (Col I staining).

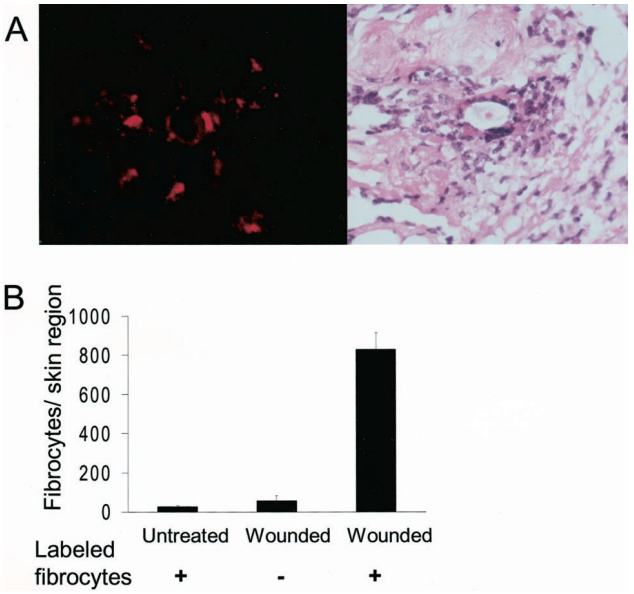

FIGURE 3. Fibrocytes migrate to wound sites in vivo. Cultured, enriched mouse fibrocytes (>96% pure) were labeled with the fluorescent dye, PKH-26. Labeled cells (5×10^5) were injected into the tail vein of BALB/c mice. Immediately following injection of the fibrocytes, a single full-thickness round skin wound was made in the dorsal subscapular area of each mouse. After 4 days mice were sacrificed, and wound sites were removed. The migration of labeled fibrocytes was assessed by fluorescent microscopic examination of thin frozen sections (*A*, *left panel*; hematoxylin-eosin staining of the wound site is shown (*right panel*)) or by quantitative cytofluorometric analysis of the number of fluorescent fibrocytes found in the biopsies of wounded skin vs nonwounded skin (with and without i.v. injection of fluorescent fibrocytes; *B*) following proteolytic dissociation of 250 μg biopsy sites.

with untreated cells. Similar results were observed with fibrocyte preparations from three other donors, each showing a 30–45% increase in Col I expression between 0 and 10 ng/ml TGF-β1.

Fibrocytes cultured ex vivo migrate to mouse wound sites

We next sought to quantify the migration into wound sites of transferred cultured, enriched fibrocytes using a mouse model system. Cultured, enriched mouse fibrocyte preparations (>96% pure) were injected (5×10^5/mouse) into the tail vein of mice. Immediately, full-thickness skin punch biopsy wounds (5 mm in diameter) were made in the dorsal scapular area in some mice. The wound sites (and comparable untreated skin tissue) were excised 4 days later, and biopsy specimens were examined for the presence of labeled fibrocytes. As shown in Fig. 3*A*, numerous fluorescent cells were found by microscopic analysis of the wound tissue at 4 days. Labeled fibrocytes appeared to be located near newly formed blood vessels at the edge of the wound. Using another group of mice ($n = 3$/group), single-cell suspensions were prepared from the excised wound or normal tissue (250 μg/biopsy), and labeled fibrocytes were quantified by flow cytometry. Enumeration of migrated labeled fibrocytes revealed that wounded tissue contained significantly more labeled fibrocytes than a similar area of normal skin taken from the same mouse (Fig. 3*B*).

Fibrocytes express functional chemokine receptors and migrate in response to SLC in vitro and in vivo

Numerous circulating cells, including, neutrophils, monocytes, and T cells, are known to migrate into cutaneous wound sites. This process is organized in part by specific interactions between chemokines and their receptors. We surveyed cultured enriched fibrocytes for chemokine receptor mRNA expression by RT-PCR and found CCR3, CCR5, CCR7, and CXCR4 mRNA (Fig. 4*A*), but not CCR4, CCR6, or CXCR3 mRNA expression. We confirmed CCR3, CCR5, CCR7, and CXCR4 protein expression on the surface of human enriched fibrocytes by flow cytometry (Fig. 4*B*). Cultured, enriched fibrocytes isolated from mouse blood also expressed CCR7 and CXCR4, as analyzed by cytofluorometric analysis (Fig. 4*C*).

Based on the expression of CCR7, a receptor for SLC, and CXCR4, a receptor for SDF, by enriched fibrocytes we used SLC and SDF in an in vitro chemotaxis assay. As shown in Fig. 5*A*, SLC significantly induced the migration of fibrocytes, whereas SDF did not. Checkerboard analyses confirmed the chemotactic (but not chemokinetic) response of cultured, enriched fibrocytes in response to SLC (Fig. 5*B*). Based on these observations, we investigated whether SLC could promote the migration of transferred, cultured, enriched fibrocytes following an i.d. injection of the chemokine in vivo. Administered at a dose of 1 μg, SLC dramatically induced the accumulation of prelabeled, ex vivo cultured fibrocytes in the skin area surrounding the i.d. injection site compared with the effect of PBS alone (Fig. 5*C*). By contrast, SDF injection did not promote fibrocyte chemotaxis in vivo (Fig. 5*C*). Immunostaining of a 2-day wound site revealed SLC chemokine expression by the vascular endothelium (data not shown). These results suggest that fibrocytes migrate into early wound sites in part due to an interaction between vascular endothelium-derived SLC and fibrocyte CCR7.

Fibrocytes contract collagen gels

Based on their presence within the wound and their expression of Col I and III, we have postulated that fibrocytes mediated wound healing and fibrosis. Gabbiani and coworkers have previously described a population of wound fibroblasts that differentiate into myofibroblasts in the presence of TGF-β (Ref. 17, reviewed in Ref. 18). These cells are characterized by expression of αSMA, the

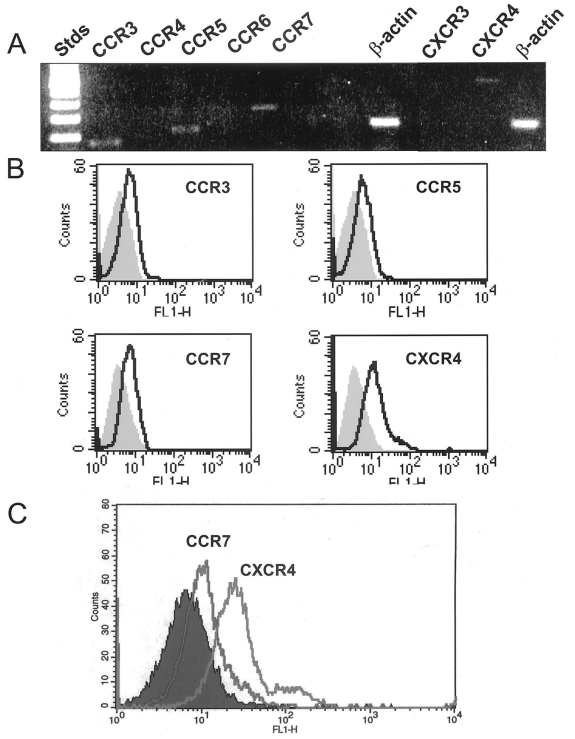

FIGURE 4. Fibrocytes express CCR3, CCR5, CCR7, and CXCR4 mRNA and protein. *A*, RT-PCR was used to determine mRNA expression for various chemokine receptors by cultured, enriched human fibrocytes. *B*, Cultured, enriched human fibrocytes were stained for surface expression with anti-CCR3, CCR5, CCR7, or CXCR4 Abs and then analyzed by flow cytometry. The shaded region represents isotype control staining. *C*, Cultured, enriched mouse fibrocytes were stained for surface expression with anti-CCR7 and CXCR4 Abs, and then analyzed by flow cytometry.

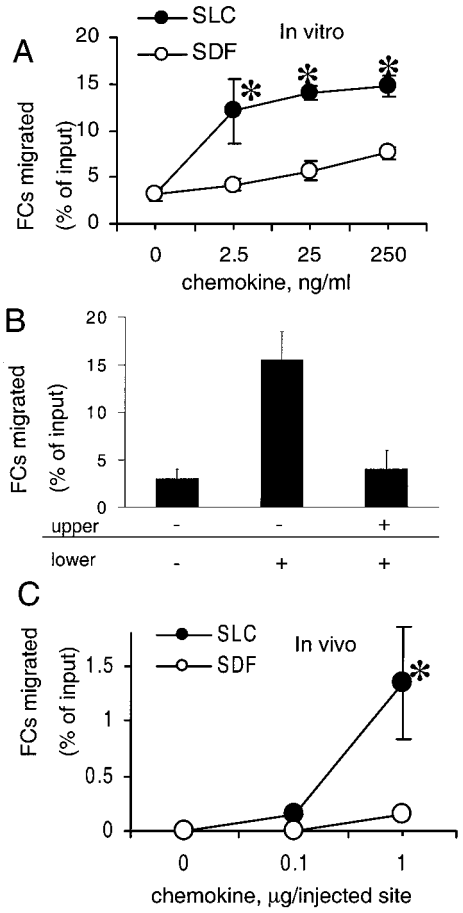

FIGURE 5. Fibrocytes migrate in response to SLC in vitro and in vivo. *A*, SLC and SDF chemokines or buffer alone diluted in DMEM/1% BSA were added to individual wells of a 24-well plate at the indicated final concentrations. Immediately thereafter, Costar Transwell devices were inserted, and cultured, enriched mouse fibrocytes (400 μl in DMEM 1% BSA at 10^6 cells/ml) were layered on top of the membrane (8 μm pore size). Cells were allowed to migrate through the membrane for 3 h at 37°C. Transmigrated cells were collected and counted by flow cytometry. The number of cells migrating to the lower chamber is presented as a percentage of the total number of fibrocytes added to the upper well. $*$, $p < 0.05$, as determined by Student's t test comparing experimental (at indicated concentrations) vs medium alone (not shown; <2%). *B*, For checkerboard-type analysis, SLC (100 ng/ml) was added to the upper and/or lower wells as indicated, and in vitro chemotaxis of fibrocytes was performed as described above. *C*, Immediately following tail vein injection of PKH-26-labeled cultured, enriched mouse fibrocytes (5×10^5), mice received an i.d. injection of SLC or SDF (0.1 or 1 μg in 50 μl) or PBS vehicle. Injected sites (250 μg) were surgically removed 4 h later and proteolytically digested to obtain a single-cell suspension. The number of labeled fibrocytes per injection site was quantified by flow cytometry and expressed as a percentage of the total number of fluorescent fibrocytes injected into the tail vein. $*$, $p < 0.05$, as determined by Student's t test, comparing SLC injected at 1 μg vs vehicle injection.

activity of contracting collagen gels in vitro, and their proposed role in wound closure, inflammation, and fibrosis (reviewed in Ref. 19). Recognizing that TGF-β1 enhances Col I expression by cultured fibrocytes (Fig. 2*D*) and that fibrocytes are present in wound tissue for days (20), we next examined whether cultured, enriched fibrocytes express αSMA and exhibit a contractile force. As shown in Fig. 6*A*, unstimulated, cultured, enriched fibrocytes were found to express αSMA mRNA, but freshly isolated PBMCs did not. Unstimulated cultured, enriched fibrocytes also express αSMA protein, and the addition of TGF-β1 (10 ng/ml) increased αSMA levels by about 4-fold (Fig. 6*B*). Next, we examined the contractile activity of cultured, enriched fibrocytes. We found that untreated cultured, enriched fibrocytes significantly contracted the collagen gels in vitro by ~20%, whereas PBMCs did not (Fig. 6*C*). Pretreatment of fibrocytes with TGF-β1 (10 ng/ml) for 7 days before the assay further increased their contractile activity (Fig. 6*C*). This increase in gel contraction by TGF-β1-treated fibrocyte cultures correlated to the enhanced expression of αSMA by fibrocytes in response to TGF-β1.

Discussion

Previous studies have shown that fibrocytes, a distinct mesenchymal cell type that arises in ex vivo cultures of peripheral blood, exhibit both monocyte- and fibroblast-like characteristics (reviewed in Ref. 21). Fibrocytes initially were identified by their rapid and specific recruitment from the blood to s.c. implanted wound chambers in mice (2). Human fibrocytes were then shown to emerge from cultures of the PBMC fraction of whole blood after 1–2 wk (2). Cultured fibrocytes have been shown to mediate fibrosis (5), Ag presentation and immunity (3, 4), and angiogenesis (C. N. Metz, unpublished observations). In the present study we have examined the differentiation pathway of peripheral blood fibrocytes and explored the role of fibrocytes in wound repair.

Fibrocytes differentiated from an adherent population of $CD14^+$-enriched peripheral blood cells when cultured in DMEM and FBS (with no additional growth factors). This differentiation process appears to require T cell interaction. Further studies will be

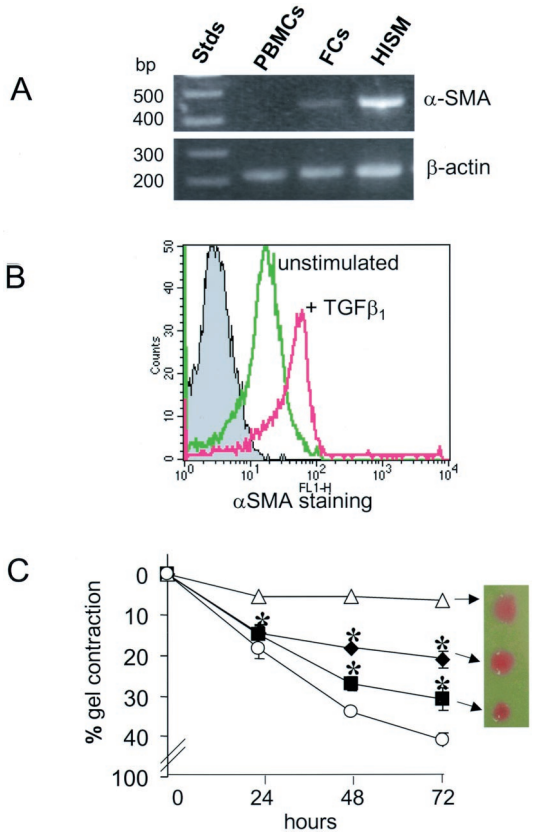

FIGURE 6. Fibrocytes express αSMA and contract collagen gels in vitro. *A*, Expression of αSMA mRNA by adherent human PBMCs, cultured, enriched fibrocytes (FCs), and human intestinal smooth muscle cells (HISM), as analyzed by RT-PCR. Stds, DNA base pair ladder. *B*, Expression of intracellular αSMA expression by unstimulated and TGF-β1 (10 ng/ml)-treated cultured, enriched human fibrocytes, as determined by flow cytometry. *C*, Collagen gel contraction assay. PBMCs (△), cultured, enriched fibrocytes (untreated (♦) and TGF-β1-treated (■)), or dermal fibroblasts (○) were resuspended in a Col I solution at 10^5 cells/ml. The contraction assay ($n = 3$) was performed as described in *Materials and Methods*. The data are percent gel contraction (from beginning of experiment) ± SE (some error bars are smaller than the symbol). ∗, $p < 0.05$, as determined by Student's *t* test, comparing experimental to PBMCs (△) at each time point. The inset shows representative contracted gels after incubation with PBMCs vs cultured fibrocytes (untreated and TGF-β1-treated).

necessary to identify the molecules involved in functionally significant interactions between T cells and fibrocytes that are required for fibrocyte maturation. Similarly, several studies have demonstrated the differentiation of $CD1a^+$ dendritic cells from GM-CSF- and IL-4-treated $CD14^+$ peripheral blood monocytes (22–25). The T cells requirement observed for fibrocyte differentiation is reminiscent of the maturation of dendritic cells, also known for their ability to process and present Ags (26). Recent studies have shown that the final step in the maturation of dendritic cells occurs during their association with $CD4^+$ T cells; this contact diminishes their responsiveness to IFN-γ by down-regulation of their IFN-γ receptors (27).

Interestingly, the addition of TGF-β1, a multifunctional cytokine that plays a central role in tissue repair and fibrosis, to crude fibrocyte-evolving cultures facilitated fibrocyte differentiation. The role of exogenous TGF-β in fibroblast proliferation and collagen production is well documented (reviewed in Ref. 28). TGF-β significantly up-regulates collagen expression by dermal fibroblasts in vitro (29), by myofibroblasts (30), as well as by proliferative scar xenografts in vivo (31). Many laboratories have confirmed that TGF-β plays a role in the natural wound healing process and that TGF-β is expressed in rodent wound chambers during the early to mid phases (days 4–7) of wound healing (32). Furthermore, in vivo gene transfer with TGF-β1 cDNA into the skin of rats significantly enhanced the rate of wound repair (33). Consistent with these prior observations, we postulate that circulating fibrocyte precursor cells interact with activated T cells, which permits their early differentiation (toward the fibrocyte phenotype), and they then migrate to the wound site (Fig. 7). Within the wound site, these early differentiated fibrocytes might further interact with recruited T cells and fully differentiate and mature following exposure to TGF-β. These fully differentiated, mature fibrocytes express increased levels of αSMA and produce collagen and other extracellular matrix proteins that promote wound healing and contracture.

Fibroblasts have been shown to exhibit increased collagen expression and other matrix components in certain fibrotic disease states (reviewed in Ref. 34). Investigators have previously implicated TGF-β overexpression in fibrosis of the skin (35) and lungs (35, 36). In addition, TGF-β overexpression has been associated with enhanced myofibroblast activity in animal models of pulmonary fibrosis (37). Our findings that TGF-β1 enhanced proliferation, collagen production, and αSMA expression by cultured fibrocytes potentially implicates this circulating cell type in TGF-β-dependent fibrotic responses in vivo.

A role for fibrocytes in wound healing and connective scar tissue formation has been postulated based on their accumulation in wound sites (2). However, the molecular signals that mediate the trafficking of fibrocytes to the wound has not yet been investigated. We examined chemokine receptor expression (mRNA and protein) by cultured enriched fibrocytes and revealed the presence of CCR3, CCR5, CCR7, and CXCR4 and the absence of CCR4, CCR6, and CXCR3. Further studies showed directed chemotaxis of cultured, enriched fibrocytes in response to the ligand of CCR7, SLC (also known as 6Ckine, Exodus-2, and TCA-4), in vitro and in vivo. SLC, a C-C chemokine family member, has been shown to be involved in the organization of lymphoid tissue during development by attracting T cells and mature dendritic cells (38). SLC expression has been observed in sites of inflammation (39). We observed SLC expression by the vascular endothelium within the wound sites. Based on these observations it would be interesting to examine the role of fibrocytes in wound responses using mutant mice lacking SLC expression (40–42).

The functional role of fibrocytes in wound healing has not been investigated previously. TGF-β has been shown to be the most important cytokine for the *trans*-differentiation of fibroblasts to contractile wound myofibroblasts that exhibit increased αSMA staining, elevated collagen secretion (reviewed in Ref. 19), and increased stress fibers (17) in response to TGF-β. Myofibroblasts are transiently found in early to mid phase wound tissue and have been proposed to exert a critical contractile force that is required to close wounds. Neither the origin of myofibroblasts nor any trafficking signals necessary for myofibroblast migration to injured

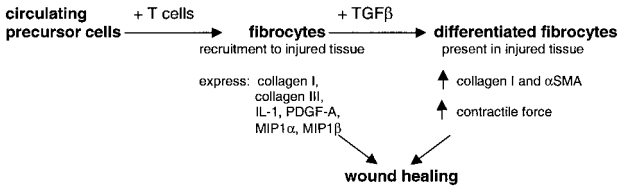

FIGURE 7. Proposed differentiation pathway of fibrocytes from a circulating precursor population.

tissue are well understood. Myofibroblasts have been postulated to derive from progenitor stem cells, resident tissue fibroblasts, or tissue smooth muscle cells. However, a plausible alternative is that myofibroblasts differentiate from a circulating, rather than a resident, precursor cell type.

In this paper we show that blood-borne, ex vivo cultured, precursor fibrocyte cells have the capacity to differentiate into αSMA^+, TGF-β1-responsive fibrocyte cells that exhibit characteristics similar to those of wound-healing myofibroblasts. Differentiated fibrocytes and myofibroblasts share many common features: transient presence within the wound, production of numerous proinflammatory cytokines and growth factors, secretion of collagen and other extracellular matrix proteins, and enhanced collagen production in response to TGF-β1. Furthermore, we observed that cultured fibrocytes, like myofibroblasts, express αSMA protein that is enhanced by TGF-β1 treatment and exert a contractile force that would aid in reducing the amount of denuded surface area of wounded tissue. The question remains of whether fibrocytes and myofibroblasts are distinct populations. However, it is reasonable to suggest that fibrocytes derived from a circulating precursor population play an important role during the resolution and repair phase of wound healing.

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