Chondrogenic and Adipogenic Potential of Microvascular Pericytes

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Background—Previous studies have shown that pericytes can differentiate into osteoblasts and form bone. This study investigated whether pericytes can also differentiate into chondrocytes and adipocytes.

Methods and Results—Reverse transcription–polymerase chain reaction demonstrated that pericytes express mRNA for the chondrocyte markers Sox9, aggrecan, and type II collagen. Furthermore, when cultured at high density in the presence of a defined chondrogenic medium, pericytes formed well-defined pellets comprising cells embedded in an extracellular matrix rich in sulfated proteoglycans and type II collagen. In contrast, when endothelial cells were cultured under the same conditions, the pellets disintegrated after 48 hours. In the presence of adipogenic medium, pericytes but not endothelial cells expressed mRNA for peroxisome proliferator–activated receptor-γ2 (an adipocyte-specific transcription factor) and incorporated lipid droplets that stained with oil red O. To confirm that pericytes can differentiate along the chondrocytic and adipocytic lineages in vivo, these cells were inoculated into diffusion chambers and implanted into athymic mice for 56 days. Accordingly, mineralized cartilage, fibrocartilage, and a nonmineralized cartilaginous matrix with lacunae containing chondrocytes were observed within these chambers. Small clusters of cells that morphologically resembled adipocytes were also identified.

Conclusions—These data demonstrate that pericytes are multipotent cells that may contribute to growth, wound healing, repair, and/or the development and progression of various pathological states. (Circulation. 2004;110:2226-2232.)

Key Words: vasculature • cardiovascular diseases • cells • microcirculation • stem cells

There is now good evidence that cells with pluripotent characteristics are present in many adult tissues, including bone marrow, skin, skeletal muscle, adipose tissue, and dental pulp. However, the identity of these cells remains to be defined. Interestingly, Bianco and colleagues recently suggested that the mesenchymal stem cells present in bone marrow might originate from microvascular pericytes. Because many of the other tissues from which pluripotent cells have been isolated are also rich in microvessels, we hypothesized that these cells may also have a vascular origin.

In support of this hypothesis, we and others have demonstrated that pericytes can differentiate into osteoblasts. Other studies have suggested that pericytes may also have the potential to differentiate into chondrocytes, adipocytes, smooth muscle (SM) cells, macrophages, and fibroblasts. However, the majority of data supporting this suggestion are indirect, and the full extent of pericyte plasticity has not been elucidated.

The purpose of the present study was to determine whether vascular pericytes could differentiate into chondrocytes and adipocytes. We demonstrate that when cultured under defined conditions, pericytes can be induced to express chondrogenic and adipogenic markers. Furthermore, we show the presence of chondrocytes and adipocytes and the formation of cartilage, fibrocartilage, and mineralized cartilage when pericytes are loaded in diffusion chambers and implanted in vivo. These data support the hypothesis that pericytes are multipotent precursor cells. The possibility that pericytes may contribute to growth, wound healing, repair, and/or the development and progression of various pathological states is discussed.

Methods

Cell Culture

Bovine retinal pericytes and aortic endothelial cells were isolated and characterized as described. Pericytes were routinely cultured in Eagle’s minimal essential medium (MEM) containing 20% fetal calf serum (FCS), 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 50 μg/mL ascorbate-2-phosphate, and nonessential amino acids (20% FCS-MEM). To induce chondrogenesis, cells were cultured in pellets. Thus, cells (5×10⁵) were resuspended in chondrogenic medium (high-glucose Dulbecco’s Modified Eagle’s Medium [DMEM] containing 10% FCS, 10⁻³ mol/L dexamethasone, 25

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µg/mL ascorbate-2-phosphate, 10 ng/mL transforming growth factor [TGF]-β3 [R&D Systems], 1 mMol/L sodium pyruvate, nonessential amino acids, and ITS+1 [fetal concentration, 10 µg/mL bovine insulin, 5.5 µg/mL transferrin, 5 ng/mL sodium selenite, 4.7 µg/mL linoleic acid, and 0.5 mg/mL bovine serum albumin [BSA; Sigma]) within a 15-mL tube and centrifuged (800g for 5 minutes). The supernatant was removed, chondrogenic medium (1 mL) was added, and the samples were centrifuged and then incubated at 37°C in a humidified atmosphere comprising 5% CO₂ and 95% air for up to 24 days. The medium was changed 3 times per week. As controls, endothelial cells were also cultured as pellets in chondrogenic medium, and pericytes were cultured as pellets in 20% FCS-MEM. Pellet wet weights were recorded, and statistical significance was determined with a Student t test. For adipocytic differentiation, confluent cells were incubated for 14 days in MEM containing 10% rabbit serum. Control cells were cultured in 20% FCS-MEM.

**Histochemical Staining and Immunostaining**

Oil red O staining was performed using standard procedures. Dual staining of cells for oil red O and either 3G5 or α-SM actin was carried out as follows. Cells were washed and incubated (1) with 3C5 (1:400), blocked in 10% formaldehyde for 10 minutes, and then incubated with 0.5% BSA/PBS/0.15 mol/L NaCl, or (2) with 4% normal goat serum, blocked with phosphate-buffered saline (PBS)/0.5% BSA/0.15 mol/L NaCl, and incubated with anti–α-SM actin antibody (1:100, 1 hour; Sigma). Cells were permeabilized in 0.1% Triton/PBS (5 minutes), blocked in 2% FCS/DMEM (30 minutes), and incubated with 2% H₂O₂ (5 minutes). Immunoreactivity was detected with rabbit anti-mouse horseradish peroxidase–conjugated secondary antibodies and diaminobenzidine. Cells were then air-dried, incubated with oil red O (15 to 20 minutes), and counterstained with Harris hematoxylin (30 seconds).

Cell pellets were fixed in 4% formaldehyde/PBS and embedded in wax. Sections (5 µm) were stained with Alcian blue and toluidine blue by standard procedures. For immunohistochemistry, sections were treated with 2% H₂O₂ (5 minutes), incubated with 0.1 U/mL chondroitinase ABC (Sigma) at 37°C (30 to 60 minutes), and blocked in 10% rabbit serum/1% BSA/PBS (1 hour). The sections were then incubated with anti–type II collagen antibody (II-H683, Developmental Studies Hybridoma Bank), anti–type I collagen antibody (MAB3391, Chemicon), or mouse IgG (all at 4.9 µg/mL for 16 hours, 4°C). For 3G5 and α-SM actin immunohistochemistry, the antigenic sites were unmasked by microwaving for 9 minutes in 0.01 mol/L citrate buffer. Immunoreactivity was detected with rabbit anti-mouse horseradish peroxidase–conjugated secondary antibodies and diaminobenzidine.

**Reverse Transcription–Polymerase Chain Reaction**

Total RNA was isolated with the use of RNA-Bee (Biogenes). Reverse transcriptase (RT) reactions were performed with and without enzyme (Roche) with 4 µg total RNA (20-µL reaction volume). Polymerase chain reaction (PCR) was performed with 5 µL of RT product, 300 mMol/L of each gene-specific primer, and 1 U Taq polymerase (Roche; 50-µL reaction volume). The conditions for amplification were as follows: 95°C (2 minutes); 35 cycles of 95°C (15 seconds), 50°C, or 55°C (30 seconds), depending on the primers, and 70°C (1 minute); and a final 7-minute extension at 70°C. The following primers were used (fragment sizes, annealing temperatures, and whether 10% dimethyl sulfoxide [DMSO] was used are indicated): aggrecan forward, GAG ACA ACG AGT TTC and reverse, ACA AAG TCT TCA CCT GTG TAG (399 bp, 50°C, 10% DMSO); type II collagen forward, GAG AAG GGA GAA GTT GGA CC and reverse, ACC ATC TTT TTC AGA AGG AC (377 bp, 55°C, 10% DMSO); Sox9 forward, CAC ATC AAG ACG GAG CAG and reverse, TGT AGA CTT GTT GTC CCC (325 bp, 55°C, 10% DMSO); and paxillin promoter–activated receptor (PPAR)-γ2 forward, CAT GGT GTA CAC AGA GAT GCC and reverse, TCA CAA GGA TGA ACT CCA TAG (380 bp, 50°C; MWG-Biotech UK). Confirmation that the correct sequences were amplified was obtained by sequencing the PCR products into the pCR 4-TOPO vector (Invitrogen) and sequencing.

**Figure 1.** Pericycle characterization. Immunofluorescence staining showing pericytes express α-SM actin (A) and antigen recognized by monoclonal antibody 3G5 (B). Nuclei are stained blue with 4',6-Diamidino-2-phenylindole. Other abbreviation is defined in text.

**In Vivo Implantation Study**

Diffusion chambers (130-µL volume) were assembled from commercially available components (Millipore UK Ltd) as described previously. Thus, chambers were placed in a Petri dish and sterilized by ultraviolet light for 1 hour. Bovine pericytes were inoculated into diffusion chambers (10⁴ to 10⁷ cells/chamber), which were sealed and then implanted intraperitoneally into 8-week-old athymic mice (Harlan, Olac, Bicester, UK) under anesthesia. Diffusion chambers, harvested 56 days after implantation, were fixed in formalin and embedded in methylmethacrylate. Sections (7 µm) were stained with toluidine blue and von Kossa’s stains.

**Results**

**Characterization of Pericytes**

Pericytes were identified by their characteristic morphology and by the expression of α-SM actin (Figure 1A) and the cell-surface ganglioside recognized by antibody 3G5 (Figure 1B).

**Differentiation of Pericytes Into Chondrocytes**

When cultured in 20% FCS-MEM, pericytes proliferate to form multicellular nodules. To determine whether these cells express chondrocytic markers, RNA was prepared from cultures containing small, nonmineralized, multicellular nodules (day 14 in culture), and RT-PCR for Sox9, type II collagen, and aggrecan was performed. Figure 2A demonstrates that these markers are expressed by pericytes. Further confirmation of the expression of chondrocyte markers by pericytes was demonstrated by the positive staining of multilayered areas and nodules with Alcian blue (Figure 2B). No staining was detected in sparse or confluent areas (Figure 2B) or after preincubation with chondroitinase ABC.

Culturing mesenchymal stem cells as high-density pellets in defined medium containing TGF-β3 induces their differentiation into chondrocytes. Therefore, to confirm that pericytes can undergo chondrogenic differentiation, cells were pelleted by centrifugation, cultured in chondrogenic medium for 24 days, and analyzed. Controls included pericycle pellets in 20% FCS-MEM and endothelial cell pellets in chondrogenic medium.

When cultured in chondrogenic medium, the pericycle pellets were significantly larger and more cellular than when cultured in normal medium (wt weights, 1.13±0.31 mg compared with 0.43±0.14 mg, respectively, P<0.005; com-
pare Figures 3A–3E and 3F–3J). Histological staining of the pericyte pellets cultured in chondrogenic medium with toluidine blue (Figure 3A) and Alcian blue (Figure 3B) revealed that they were composed of cells embedded within a proteoglycan-rich extracellular matrix. Immunohistochemical staining demonstrated that type II collagen was present throughout the matrix of the pellets (Figure 3C). Weak staining on the periphery of the pellets was observed with antibodies to type I collagen (Figure 3D) and 3G5 (Figure 3K). Many of the cells within the pellets expressed α-SM actin (Figure 3L). Pericyte pellets cultured in normal medium did not stain metachromatically with toluidine blue (Figure 3F) and were only weakly positive for Alcian blue (Figure 3G), suggesting that only minor amounts of cartilage proteoglycans were deposited. Furthermore, type I collagen but not type II collagen was detected in the matrix of these pellets (Figures 3I and 3H). No staining was detected in the negative controls (Figures 3E and 3J). When endothelial cells were cultured as pellets in chondrogenic medium, the pellets disintegrated after 48 hours.

To confirm that pericytes form a cartilaginous matrix, they were inoculated into diffusion chambers, implanted into athymic mice for 56 days, and examined. Figure 4 shows 2 examples of tissue formed within the chambers. Mineralized cartilage was localized adjacent to the Millipore filter, and lacunae containing chondrocytes were observed within the

Figure 2. Expression of chondrocytic markers by monolayer pericytes. A, RT-PCR analysis of gene expression in bovine retinal pericytes cultured for 14 days in 20% FCS-MEM. PCR was performed with primers for aggrecan (399 bp), type II collagen (377 bp), and Sox9 (325 bp). Controls (−RT) are also shown. B, Postconfluent pericytes stained with Alcian blue. Bar=150 μm. All abbreviations are as defined in text.

Figure 3A–3F. Chondrogenic differentiation of pericytes is promoted by culturing cells as pellets in chondrogenic medium. Pericyte pellets were cultured in chondrogenic medium (A–E, K, L) or control medium (F–J). Cell nuclei are stained with hematoxylin. Sections stained with toluidine blue (A, F) and Alcian blue (B, G). Sections stained for type II collagen (C, H), type I collagen (D, I), 3G5 (K), and α-SM actin (L). Arrows indicate 3G5-positive cells (K); open arrows indicate α-SM actin-negative cells (L). E, J, Control sections incubated with mouse IgG. Bar=200 μm. All abbreviations are as defined in text.
cartilaginous matrix adjacent to the mineralized cartilage (Figure 4A). Areas resembling fibrocartilage were localized toward the centers of the chambers (Figure 4B).

Differentiation of Pericytes Into Adipocytes

The differentiation of mesenchymal stem cells and osteoblasts into adipocytes is induced by culturing the cells in rabbit serum. Therefore, to investigate whether pericytes could differentiate into adipocytes, cells were cultured in 10% rabbit serum-MEM for 14 days. Pericytes incubated in 20% FCS-MEM served as controls. In addition, endothelial cells were incubated in medium containing rabbit serum or FCS. Adipocytic differentiation was assessed by staining the cells with oil red O and by examining the expression of PPAR-γ2. Figure 5A shows that some but not all pericytes cultured in the presence of rabbit serum contained intracellular lipid droplets that stained with oil red O. In contrast, no staining was observed in controls (Figure 5B) or in endothelial cells incubated in either rabbit serum or FCS. To determine whether cells containing lipid droplets retained their pericytic phenotype, dual staining was performed. Figure 5 demonstrates that there was no correlation between the expression of pericyte markers and the deposition of lipid droplets. Thus, cells containing both 3G5 and lipid droplets (Figure 5C) and α-SM actin and lipid droplets (Figure 5D) were observed in addition to cells containing only one of these markers. To confirm the adipocytic identity of pericytes cultured in rabbit serum, the presence of mRNA for PPAR-γ2 was demonstrated by RT-PCR (Figure 5G). PPAR-γ2 mRNA was not detected when RT was omitted, when endothelial cells were incubated in rabbit serum (Figure 5G), or when cells were incubated in FCS (data not shown). When pericytes were loaded in diffusion chambers and implanted into athymic mice, small clusters of cells that morphologically resembled adipocytes were identified adjacent to the Millipore filters (Figure 4B).

Discussion

Previous studies have shown that microvascular pericytes can form bone in vitro and in vivo. We now demonstrate that pericytes can also differentiate into chondrocytes and adipocytes. These studies support the hypothesis that adult pericytes may serve as a reservoir of primitive precursor cells and as such, may contribute to growth, wound healing, repair, and the development and progression of various pathological states, including vascular calcification and the formation of chondrogenic metaplasia in these vessels.

We first demonstrated that postconfluent cultures of pericytes express chondrogenic markers (Sox9, collagen II, and aggrecan). Furthermore, the deposition of a matrix rich in sulfated proteoglycans within the nodules formed in these cultures was confirmed by their positive staining with Alcian blue. This is the first time that Sox9, a transcription factor that has an essential role in chondrocyte differentiation, has been identified in pericytes. Therefore, these results support and extend previous studies in which the expression of mRNA for aggrecan and type II collagen was demonstrated in pericytes. We then demonstrated that by culturing these cells at high density as pellets in the presence of a defined, chondrogenic medium containing TGF-β3, pericytes deposited a cartilaginous matrix enriched in sulfated proteoglycans and type II collagen, reminiscent of the matrix found in articular cartilage. Type I collagen was also detected at the periphery of the pellets, where the cells were elongated, resembling the morphology of fibroblastic cells. These data are in accord
with studies that have demonstrated the formation of similar structures when chondrocytes\(^1\) and human bone marrow mesenchymal stem cells are cultured as pellets.\(^{12,16}\) Indeed, Mackay and colleagues\(^{12}\) hypothesized that the interior of the pellets provides an environment that promotes the chondrogenic differentiation of mesenchymal stem cells, whereas the periphery of the pellets is more amenable to fibroblastic or synovial differentiation.

We also demonstrate that specific growth factors and a high cell density are important for inducing the chondrogenic phenotype in pericytes. Thus, in normal growth medium, the pericyte pellets were significantly smaller and less cellular than when grown in chondrogenic medium. Furthermore, the matrix comprised mainly type I collagen, with very little type II collagen or sulfated proteoglycans being deposited. These data are consistent with previous studies demonstrating that high-glucose medium and TGF-β3 are important for chondrogenic differentiation of mesenchymal stem cells.\(^{12}\)

The demonstration that many of the cells within the pellets expressed α-SM actin and that cells on the periphery of the pellets stained with 3G5 antibodies suggests that these pericytes may not be fully differentiated into chondrocytes. Furthermore, it is noteworthy that not all cells deposit a cartilaginous matrix when they are cultured as pellets in chondrogenic medium. Thus, we have shown that endothelial cells do not form compact pellets in culture. Instead, the structures disintegrate within 48 hours. In addition, although fibroblasts form pellets in culture, a cartilaginous matrix was not deposited.\(^{16}\) Together, these results suggest either that not all cells have multilineage potential or that the conditions required to fully induce the chondrogenic differentiation of these cells remains to be defined.

Loading the cells in diffusion chambers and implanting them intraperitoneally in athymic mice provided the final proof that pericytes have chondrogenic potential. These chambers have previously been used to demonstrate the potential of bone marrow mesenchymal stem cells to form bone and cartilage.\(^{20–22}\) Importantly, neither bone nor cartilage is formed when endothelial cells or cells isolated from other nonskeletal sites are implanted in these chambers.\(^{20,22,23}\) Previously, we demonstrated that bone, mineralized bone, and cartilage are formed when pericytes are loaded in these chambers.\(^7\) In this article, we demonstrate that fibrocartilage, nonmineralized cartilage, and mineralized cartilage are also...
formed. In addition, lacunae containing chondrocytes were observed. Our data extend previous studies demonstrating that vascular cells contribute to neochondrogenesis in grafted perichondrium.24 However, that latter study did not demonstrate whether the progenitors were pericytes or endothelial cells.

The results presented herein also demonstrate for the first time that pericytes can differentiate into adipocytes. Thus, cells resembling adipocytes were detected when pericytes were loaded in diffusion chambers. In addition, when pericytes were cultured in the presence of rabbit serum, mRNA for the adipocyte-specific transcription factor PPAR-γ2 was detected. In addition, some but not all of the cells incorporated lipid droplets that stained with oil red O. However, the retention of pericyte markers by many of the cells containing lipid droplets suggests that these cells may not be fully differentiated into mature adipocytes. The conditions needed to induce the differentiation of pericytes into mature adipocytes in vitro remain to be defined. In contrast, neither PPAR-γ2 mRNA nor oil red O–positive lipid droplets were detected when endothelial cells were cultured under the same conditions, suggesting that not all cells can differentiate into adipocytes. Our data are consistent with previous studies demonstrating the interrelationship between adipocyte, osteoblast, and chondrocyte lineages.1,3 Indeed, osteoporosis is characterized by decreased osteogenesis and increased adipogenesis of bone marrow mesenchymal cells and increased osteogenesis in blood vessels.25

So, why is it interesting that pericytes have multilineage potential? First, a continuous subendothelial network of pericyte-like cells has been identified throughout the entire human vascular bed.26 Second, pericytes may play a role in normal growth and development. For example, it is well established that bone formation is suppressed when angiogenesis is inhibited.27,28 Therefore, because pericytes are an essential part of the angiogenic process, these cells may directly contribute to skeletogenesis. Third, pericytes may serve as a reservoir of primitive precursor cells. Indeed, there are many phenotypic similarities between pericytes and stem cells isolated from adult tissues1–3,7,29 (and this article). Finally, pericytes may be involved in the development and progression of several pathological conditions, including vascular calcification.30,31 Indeed, markers of both cartilage and bone have been identified in calcified blood vessels,31,34–36 and it has been suggested that a subpopulation of SM cells that resemble pericytes (calcifying vascular cells) contributes to this calcification.30–32 A recent study has shown that these cells also have multilineage potential in vitro.36

To conclude, we have shown that pericytes can differentiate into osteoblasts, chondrocytes, and adipocytes both in vitro and in vivo. However, it still remains to be determined whether these cells can also differentiate into other cell types, such as myoblasts or neural cells. In addition, we still need to understand how pericyte differentiation is regulated (i.e., what keeps pericytes as pericytes in a normal blood vessel, and what stimulates them to differentiate along specific lineages?). We have already begun to address these questions and have identified several factors (e.g., Axl and matrix Gla protein) that regulate pericyte differentiation along the osteogenic pathway,14,15 and studies are in progress to determine the molecular mechanisms regulating their differentiation into chondrocytes.

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