Osteoarthritis and Cartilage xxx (2016) 1–8

Osteoarthritis and Cartilage

Enhanced phagocytic capacity endows chondrogenic progenitor cells with a novel scavenger function within injured cartilage

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ARTICLE INFO

Article history:
Received 13 July 2015
Accepted 19 April 2016

Keywords:
Chondrogenic progenitor cells
Phagocytosis
Chondrocytes
Synoviocytes
Macrophages

SUMMARY

Objective: Articular cartilage harbors chondrogenic progenitor cells (CPCs), a population that responds chemotactically to cell death. Because this behavior is reminiscent of macrophages, we hypothesized that CPCs have macrophage-like capabilities for scavenging cell and tissue debris through phagocytosis.
Design: CPCs, chondrocytes, synoviocytes, and macrophages were cultured with fluorophore-labeled chondrocyte debris for 3, 6, 12, or 24 h. Debris internalization was quantified by confocal microscopy and flow cytometry. Confocal microscopy was also used to test CPCs and chondrocytes for uptake of fluorophore-labeled fibronectin fragments (Fn-fs), a form of extracellular matrix debris. Lysosome activity and mass in CPCs and chondrocytes were measured using fluorescent probes. The relative expression of phagocytosis-related genes and proteins was evaluated by polymerase chain reaction (PCR) and immunoblotting, respectively. Pulse-chase experiments were performed to determine if the debris internalized by CPCs and chondrocytes was cleared, and if clearance was affected by a cathepsin B inhibitor.
Results: More macrophages, synoviocytes, and CPCs internalized cell debris than chondrocytes at all time points. While uptake remained flat in chondrocytes at ~10%, in the other cell types it peaked at more than 60% after 12–24 h. Relative to chondrocytes, CPCs showed significantly higher rates of Fn-fs engulfment, greater lysosome activity and mass, and over-expressed phagocytosis-related genes and proteins. Pulse-chase experiments revealed time- and cathepsin B-dependent clearance of cell debris in CPCs, but not in chondrocytes.
Conclusions: CPCs phagocytized cell and matrix debris much more efficiently than chondrocytes, supporting the hypothesis that they play a macrophage-like role in injured cartilage.

Introduction

The long-held belief that articular cartilage harbors only one cell type, the chondrocyte, has given way in the face of evidence for a second distinct type, the chondrogenic progenitor cell (CPC), which has been identified in healthy, injured, and osteoarthritic cartilage[1–6]. Under normal circumstances CPCs are thought to represent less than 1% of cartilage cells. However, our previous work documented the emergence and proliferation of CPCs on cartilage surfaces after a mechanical injury, which leads to several fold increases in their numbers in and near the sites of tissue damage and chondrocyte death[6]. These cells were found to migrate to injury sites along alarmin and chemokine gradients, a chemotactic behavior that chondrocytes do not perform. However, like superficial chondrocytes, CPCs express relatively high levels of lubricin, a boundary lubricant that protects cartilage from mechanical damage[6,7]. Migrating CPCs are morphologically distinguished from non-motile chondrocytes by their elongated and sometimes dendritic shape, which contrasts sharply with the typically spherical chondrocytes. CPCs express some, but not all of the markers used to identify MSCs. The overall pattern of CPC gene expression revealed by microarray analysis is more akin to synovial fibroblasts and mesenchymal stem cells than chondrocytes[8]. Unlike pluripotent MSCs, CPCs are limited in their ability to form different tissues; however, as their name implies, CPCs reliably generate hyaline cartilage under chondrogenic conditions[9].

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http://dx.doi.org/10.1016/j.joca.2016.04.016
1063-4584/

Phagocytosis is described as the engulfment and destruction of extracellular objects and substances by cells. Engagement of cell-surface receptors by extracellular ligands leads to internalization via phagosomes and endosomes, followed by lysosomal degradation, a process that depends on proteases, which include cathepsin B, the most abundant cathepsin in lysosomes[10]. Phagocytosis plays a prominent role in the immune response to tissue damage, where it contributes to the clearance of pro-inflammatory necrotic tissue and cell remnants, a vital tissue repair function supported mainly by macrophages. Cell-death-related ligands serve as chemokines that lure circulating monocytes to wounds, where they become mobile macrophages[11,12]. While many different cell types, including synoviocytes, are capable of chemotaxis and phagocytosis, macrophages are specialists that relocate and scavenge debris with particular alacrity. This occurs regularly throughout vascularized tissues, but the peculiar anatomy of synovial joints may thwart the ability of macrophages to reach damaged cartilage in large numbers, in that most chondral lesions are physically remote from the synovium, where sentinel macrophages and synoviocytes are stationed and blood vessels offer an ongoing supply of circulating monocytes. Supporting this conclusion are data indicating that synovial inflammation in joints with isolated chondral injuries is relatively mild[13].

The observations cited above point to a potential deficiency in debris clearance as a limitation to spontaneous cartilage repair. Chondrocytes isolated from osteoarthritic patients show some phagocytic activity[14], but their ability to clear cell debris at injury sites is weakened by their unresponsiveness to injury-related chemokines[6]. The lack of other extrinsic scavenging mechanisms led us to consider whether CPCs could provide the required phagocytic activities. As an initial test of this hypothesis we used flow cytometry and confocal microscopy to compare CPCs to chondrocytes with respect to the uptake of fluorescently-labeled cell and extracellular matrix debris. Fn-fs were chosen to represent matrix debris despite the fact that fibronectin is a minor component of the cartilage matrix (<1%). This was based on observations that Fn-fs drive local chondrolysis in injured cartilage, which makes them a high-priority target for wound-site clearance[15]. Chondrocytes were isolated from full-thickness cartilage (including the superficial, transitional, and deep zones), or from the top third of the matrix (including the superficial and upper transitional zones). Macrophages and synoviocytes, which are known to be phagocytic, were included in the cell debris uptake studies. Lysosomal activity in CPCs and chondrocytes was measured by flow cytometric analysis of intracellular cathepsin activity. A cathepsin B inhibitor was used to assess the role of lysosomal proteases in the clearance of DiO-labeled cell debris. The relative expression of phagocytosis markers in CPCs and chondrocytes was evaluated by polymerase chain reaction (PCR) and immunoblotting.

Method

Cartilage harvest and culture

Fresh articular cartilage was harvested from bovine tibial plateaus of healthy stifle joints (Bud's Meat, Riverside, IA). The cartilage was then cultured in standard media (1:1 mixture of DMEM and F12, supplemented with 10% fetal bovine serum, 50 μg/ml L-ascorbate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml Fungizone).

Cell isolation and culture

CPC detection and isolation was performed as previously described [Fig. 1(A)][6]. Briefly, a sterile 18 G needle was dragged on the cartilage surface of each explant to create multiple matrix tears and induce chondrocyte death. After 10-days in culture, the emergence of CPCs on the injured cartilage surface was then confirmed. Briefly, explants were washed with Hanks' Balanced Salt Solution and stained with 1 μM Calcein Green-AM (Invitrogen, Grand Island, NY) for 30 min, followed by confocal microscopy detection. For CPC isolation, each explant surface was treated with 0.25% Trypsin-EDTA (Gibco, Grand Island, NY) for 10 min, culture media was added to end trypsinization, and cell suspension was then centrifuged at 300 G for 10 min. Cells were resuspended and seeded in multiple 35 mm dishes. After isolation of CPCs, the underlying cartilage tissue was shaved off the subchondral bone, minced into smaller pieces, and digested in 0.03% collagenase/protease (dissolved in culture media) for 16 h. The digestion media was then centrifuged (300 G for 10 min) and resuspended, cells were seeded into multiple 35 mm dishes. To determine if phagocytic cells were enriched in the superficial zone, chondrocytes from the upper 1/3 cartilage were separated from the bottom 2/3 prior to cell isolation using a customized fixture[9]. All cells were allowed to adapt to culture conditions for at least 2 days before measuring phagocytic activities.

Synovium tissue was obtained from fresh bovine knee joint, then minced to smaller pieces to attach on culture dishes. The culture media was added onto the synovium tissue drop by drop after couple hours of dry attachment. Small amount of culture media was replenished on the following day until the synovium tissue was no longer attached. Synoviocytes were collected for experiments at passage 1.

The mouse macrophage cell line (RAW 264.7) was a generous gift from Dr Wendy Maury (The University of Iowa). Macrophages were cultured in macrophage-specific culture media (DMEM, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml Fungizone).

Generation of DiO-labeled cell debris

The lipophilic fluorescent tracer 3-octadecyl-2-[3-(3-octadecyl-2(3H)-benzoxazolylidene)-1-propenyl]-, perchlorate (DiO) was used to label lipids and lipophilic proteins in freshly isolated chondrocytes[14]. Cells were resuspended at 10^6 cells/ml in culture media, and DiO solution (Molecular Probes, Eugene, OR) was then added at 5 μl/ml. After 20 min of incubation at 37°C, the mixture was centrifuged (1500 rpm for 5 min) and resuspended in 2 ml culture media (around 1×10^7 cells), 10 freeze-thaw cycles (liquid nitrogen and 37°C water bath for 20 min, alternatively) were applied to generate the DiO-labeled cell debris.

Generation of FITC-labeled fibronectin fragments (Fn-fs)

Human fibronectin fragments (Fn-fs) mixture, containing Fn-fs of 29 kDa, 40–60 kDa and 120–160 kDa, (generated by Dr Gene Homandberg) was labeled by fluorescein isothiocyanate (FITC). Briefly, Fn-fs solution was dialyzed against 0.1 M $NaHCO_3$ for 6 h, then incubated with FITC stock solution (3 mg/ml) at a ratio of 1:170 (Fn-fs: FITC) by mass for 2 h at room temperature. FITC conjugated Fn-fs solution was then dialyzed exhaustively against 1× PBS to remove excess materials.

Detection and quantification of phagocytosis + cells

After 1 day in culture, DiO-labeled cell debris (100 μl) and FITC-labeled Fn-fs (10 μg) were added to each dish (35 mm). The cells were then cultured for various time periods (3 h, 6 h, 12 h, and 24 h). For microscopy analysis, cells were washed with Hanks' Balanced Salt Solution and stained with 1 μM Calcein Red-Orange

Please cite this article in press as: Zhou C, et al., Enhanced phagocytic capacity endows chondrogenic progenitor cells with a novel scavenger function within injured cartilage, Osteoarthritis and Cartilage (2016), http://dx.doi.org/10.1016/j.joca.2016.04.016