Age-related Biological Characterization of Mesenchymal Progenitor Cells in Human Articular Cartilage

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Abstract

Full article available online at ORTHOSuperSite.com. Search: 20110627-06

Adult articular cartilage has a low regeneration capacity due to lack of viable progenitor cells caused by limited blood supply to cartilage. However, recent studies have demonstrated the existence of chondroprogenitor cells in articular cartilage. A critical question is whether these mesenchymal progenitor cells are functionally viable for tissue renewal and cartilage repair to postpone cartilage degeneration.

This study was designed to compare the number and function of mesenchymal progenitor cells in articular cartilage collected from human fetuses, healthy adults (aged 28-45 years), and elderly adults (aged 60-75 years) and cultured in vitro. We detected multipotent mesenchymal progenitor cells, defined as CD105+/CD166+ cells, in human articular cartilage of all ages. However, mesenchymal progenitor cells accounted for 94.69±2.31%, 4.85±2.62%, and 6.33±3.05% of cells in articular cartilage obtained from fetuses, adults, and elderly patients, respectively (P<.001). Furthermore, fetal mesenchymal progenitor cells had the highest rates of proliferation measured by cell doubling times and chondrogenic differentiation as compared to those from adult and elderly patients. In contrast, alkaline phosphatase levels, which are indicative of osteogenic differentiation, did not show significant reduction with aging. However, spontaneous osteogenic differentiation was detected only in mesenchymal progenitor cells from elderly patients (with lower Markin scales). The lower chondrogenic and spontaneous osteogenic differentiation of mesenchymal progenitor cells derived from elderly patients may be associated with the development of primary osteoarthritis. These results suggest that measuring cartilage mesenchymal progenitor cells may not only identify underlying mechanisms but also offer new diagnostic and therapeutic potential for patients with osteoarthritis.
Osteoarthritis results from an imbalance between damage to and repair of articular cartilage. Chondrocytes are thought to be terminal cells with limited capacity for proliferation, primarily because of poor blood circulation and a limited number of available stem cells. The articular cartilage is, therefore, often considered to be at high risk for age-related diseases. However, several recent studies have determined that chondrocytes from healthy primary osteoarthritis patients express markers specific for stem cells. These cells are called mesenchymal progenitor cells. Why, then, can’t the existing mesenchymal progenitor cells prevent or postpone cartilage degeneration? Previous studies have shown that aging stem cells from the bone marrow reduce the rate of generation and have shown that aging stem cells from the cartilage degeneration? Previous studies have determined that chondrocytes from healthy primary osteoarthritis patients express markers specific for stem cells. These cells are called mesenchymal progenitor cells.

We hypothesized that the ability of cartilage mesenchymal progenitor cells to proliferate and differentiate declines with age, leading to age-related decrease in chondrocyte numbers and function. This age-related decline in chondrocyte number and function directly contributes to the development of primary osteoarthritis. This study compared the in vitro functions of mesenchymal progenitor cells, defined as CD166+/CD105+ cells, isolated from cartilage and surgically removed from patients of different ages. The results may offer new insights into the pathogenesis of osteoarthritis and lead to the development of new preventive and therapeutic agents to improve the quality of life for patients with osteoarthritis.

**Materials and Methods**

**Collection of Articular Cartilage**

Fetal cartilage samples were obtained from fetuses aged 20 to 24 weeks who had died of congenital heart abnormalities. Samples of knee joint cartilage were taken from adult and elderly patients who had either died of other diseases or had undergone limb amputation. All cartilage was without visible joint disease (Table 1). Articular cartilage was dissected from the femoral condyle with perichondrium and subchondral bone excised and washed 3 times with phosphate buffered saline to remove blood and soft tissue on the surface. All samples were graded according to a modified Mankin scale (0-2). Despite our efforts in choosing cartilage with minimal evidence of degeneration, cells collected for the study may have had age-associated degenerative changes in cartilage, which may have impacted the study. Samples were obtained after an informed consent form was signed by the patients and/or guardians. The study was approved by the hospital ethics committee.

**Culture of Cartilage Cells**

Articular cartilage cells were isolated as previously described. Briefly, an articular cartilage was cut into 1-mm³ pieces and incubated with phosphate buffered saline containing 0.1% trypsin, 0.1% hyaluronidase, and 0.2% type II collagenase at 37°C for 1 hour and 3 hours, respectively. Chondrocytes were harvested by centrifugation at 1000 rpm for 5 minutes and seeded into a cell culture flask at a plating density of 3×10⁴ cells/cm² in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) medium with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The cell culture was maintained at 37°C with air that contained 5% (v/v) CO₂. The medium was changed 72 hours after initial seeding, and chondrocytes continued to be cultured until 80% confluent. Cells were then detached with trypsin (0.25%) and reseeded at control densities for functional assays.

**Detection of CD105+/CD166+ Cells in Chondrocytes From Articular Cartilage**

Cells from the primary culture and after the second and fourth passages were detached with trypsin and resuspended to a final concentration of 105 cells/50 μl in phosphate buffered saline supplemented with 1% bovine serum albumin and incubated with phycoerythrin-conjugated anti-CD105 (monoclonal antibody SN6) and fluorescein isothiocyanate (FITC)-conjugated anti-CD166 antibodies for 45 minutes at 4°C. Cells stained with a mouse isotype IgG were used as negative control. After antibody binding, cells were washed 3 times with phosphate buffered saline containing 1% bovine serum albumin to remove unbound antibodies and fixed in 4% paraformaldehyde in phosphate buffered saline for 15 minutes at room temperature. They were subjected to fluorescence-activated cell sorting us-

<table>
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<tr>
<th>Group</th>
<th>Patient Sex</th>
<th>No. Patients</th>
<th>Patient Age</th>
<th>Disease</th>
<th>Scale</th>
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<tbody>
<tr>
<td>Fetal</td>
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<td>Congenital cardiac malformation</td>
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<tr>
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<td>28-45 y</td>
<td></td>
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<tr>
<td>Elderly</td>
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<td>65.8±5.5 y</td>
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<td>F</td>
<td>3</td>
<td>60-75 y</td>
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and then detected for specific antibody
ters to exclude debris and cell aggregates
first gated on the forward and side scat-
e384
an FITC-CD166 antibody (1:50 dilution)
buffered saline. They were incubated with
harvested and suspended in phosphate
from the fourth passage in culture were
bach, Germany). Briefly, chondrocytes
facturer (Miltenyi Biotec, Bergisch Glad-
according to instructions from the manu-
minimACS separation system were used
Isolation of Articular Cartilage

Isolation of Articular Cartilage Mesenchymal Progenitor Cells

An Anti-FITC MultiSort Kit and a
miniMACS separation system were used to
purify mesenchymal progenitor cells according to instructions from the manu-
facturer (Milltenyi Biotec, Bergisch Glad-
bach, Germany). Briefly, chondrocytes from the fourth passage in culture were
harvested and suspended in phosphate buffered saline. They were incubated with
an FITC-CD166 antibody (1:50 dilution)
for 45 minutes in the dark at 4°C. The
stained cells were washed twice and then
resuspended in 80 μl of 1× phosphate
buffered saline containing 1% bovine se-
rum albumin and 0.1% Na3N. They were
then incubated with 10 μl of an anti-FITC
antibody coupled to magnetic beads for 30
minutes at 4°C. After washing, CD166+
cells were selected with a magnetic ap-
paratus (Milltenyi Biotec). The selected
CD166+ cells were further selected with
CD105 micromagnetic beads (Milltenyi
Biotec) to eventually obtain cells that
are positive for CD166 and CD105. The
sorted cells were cultured at a density of
5×104 cells/cm².

Laser Scanning Confocal Fluorescence Microscopy

Magnetic bead-purified mesenchymal progenitor cells were either prepared as
smears for histological examination or seeded on a 10×10-mm cover glass to be
cultured in DMEM/F-12 culture medium for 3 days. Cells prepared in both tech-
niques were fixed in 4% polyoxymethylene (30 minutes at room temperature). The
fixed cells were first blocked with a
buffer containing 5% fetal bovine serum and 2% bovine serum albumin for 30 min-
utes and then stained for phycoerythrin-
conjugated CD166 (1:50 dilution) and
FITC-conjugated CD105 (1:50 dilution) antibodies (phycoerythrin or FITC mouse
IgG as controls) for 30 to 45 minutes at
room temperature. The slides were counter-
stained with 4,6-diamidino-2-phenylindole (DAPI) to mark the nucleus. Fluores-
cently labeled cells were visualized and imaged under a confocal laser scanning
microscope (Leica TCS SP5; Leica Mi-
crosystems, Wetzlar, Germany).

Cell Doubling Time

Cell doubling time (Td) was used to
calculate the ability to proliferate. Mes-
enchymal progenitor cells in culture (sec-
ond passage) were detached with trypsin
(0.25%), suspended in DMEM/F-12 me-
dium containing 10% fetal bovine serum,
and plated into a 96-well plate (2×103
cells/well). Cells in 3 wells from each age
group were processed in a standard MTT
procedure10 and examined every 24 hours
(optical density, 570 nm). A growth curve
was plotted from these optical density
values to calculate mesenchymal progeni-
tor cells-cell doubling time in each age
group based on the following formula:
Td=t*lg2/(lgNt-lgN0), where t is time in
culture (hours), N0 is the seeding density
of cells, and Nt is the cell density after t
hours in culture.

Detection of Articular Cartilage Mesenchymal Progenitor Cells Differentiation

Chondrogenic Induction. Mesenchy-
mal progenitor cells were cultured with
chondrogenic medium (DMEM/F-12
supplemented with 1% ITS Liquid Media
Supplement (Sigma-Aldrich Co, St Louis,
Missouri), 1 mM sodium pyruvate, 37.5 g/
ml ascorbate 2-phosphate, 10-8 M dexa-
methasone, and 10 ng/mL recombinant
human transforming growth factor-β1)
for 2 weeks.4 Control cells were cultured
in DMEM/F-12 containing 10% fetal bo-
vine serum.

After 2 weeks in culture, total RNA
was isolated using TRIzol reagent (Tian-
gen Biotech Co Ltd, Beijing, China) and
used for semiquantitative reverse trans-
scription-polymerase chain reaction to
detect the expression of type II collagen
and aggrecan gene. Reverse transcription
was first performed with 1 μg of total
RNA from each sample using oligo(dT)18
primer and 200 units of SuperScript II RT
(Life Technologies Inc, Gaithersburg,
Maryland) for cDNA synthesis. DNA (in triplicate) was then amplified in 20 μL solution that contained 2 μL diluted template, 10 pmol primer pairs for type II collagen, and aggrecan and control glyceraldehyde 3-phosphate dehydrogenase (Table 2), respectively, and 10 μL Taq PCR Master Mix (TianGen Biotech Co Ltd). The amplification was induced first at 94°C for 5 minutes, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 57°C, and 30 seconds at 72°C. The reaction was completed by a final incubation at 72°C for 10 minutes. Gene expression was expressed as 2^{-ΔΔ(Ct)}, where Ct is the cycle threshold, ΔCt of the tested gene Ct of glyceraldehyde 3-phosphate dehydrogenase, and ΔΔCt is the ΔCt of sample 1 – ΔCt of sample 2.11

Osteogenic Induction. Mesenchymal progenitor cells (1×10^4/cm²) were first cultured in DMEM/F-12 medium for 24 hours and then switched to the osteogenic medium (50 μmol/L ascorbic acid, 10 μM β-sodium glycerophosphate, and 0.1 μM dexamethasone)12 for 14 days. Control cells were cultured in DMEM/F-12 containing 10% fetal bovine serum. Intracellular alkaline phosphatase was measured with a commercial kit (LabAssay; Wako Pure Chemical Industries, Ltd, Osaka, Japan) at 405 nm and calculated as instructed by the manufacturer.

Statistical Analysis
All values are presented as mean±standard error of mean from repeated experiments. The quantitative data were analyzed using SPSS 13.0 software (SPSS, Inc, Chicago, Illinois). The Kruskal-Wallis test (non-parametric) was used to compare among multiple groups. The independent samples t test was used to assess difference between 2 groups of variables. A P value <.05 was considered statistically significant.

RESULTS
CD105+/CD166+ Cells in Articular Cartilage
The percentage distributions of CD105+/CD166+, CD34+, and CD45+ cells purified from articular cartilage were similar to mesenchymal progenitor cells from the bone marrow (Figure 1), consistent with a previous report on mesenchymal progenitor cells.4 In primary culture, articular cartilage from fetuses had the highest percentage of CD166+/CD105+ cells as compared to those from adult and elderly patients (P<.001). There was no statistical difference in the counts of CD166+/CD105+ cells between articular cartilage obtained from adult and elderly patients. However, the percent of CD166+/CD105+ cells from adult and elderly patients significantly increased in culture, whereas those from fetal tissue (which counted for >90%) did not show significant changes (Table 3; Figure 2).

Growth and Proliferation of Mesenchymal Progenitor Cells
The mesenchymal progenitor cells from fetal articular cartilage were short, spindle-shaped cells that grew rapidly, whereas most of the mesenchymal progenitor cells from articular cartilage of adult and elderly patients were longer spindle-shaped cells (Figure 3). The proliferation capacity was lower for mesenchymal progenitor cells from adult and
elderly patients as compared to those from fetal samples, with the cell doubling times of 25.68±7.71 hours, 45.35±15.41 hours, and 55.69±16.52 hours for fetal-, adult-, and elderly-derived mesenchymal progenitor cells, respectively (Figure 4).

**Chondrogenic Differentiation**

mRNA for aggrecan and type II collagen were the highest in chondrogenic-induced fetal mesenchymal progenitor cells as compared to those from adult and elderly patients (P<.05) (Figure 5). These

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>Fetal</th>
<th>Adult</th>
<th>Elderly</th>
<th>P Value</th>
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<tr>
<td>Primary cells</td>
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<td>4.85±2.62</td>
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<tr>
<td>P2 cells</td>
<td>93.18±3.11</td>
<td>11.35±3.81</td>
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<tr>
<td>P value</td>
<td>.346</td>
<td>&lt;.001</td>
<td>.008</td>
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*Mesenchymal progenitor cells in fetal cartilage as compared to adult and elderly patients. The adult and elderly groups showed no statistical difference.

Figure 2: Fluorescence-activated cell-sorting analysis of chondrocytes was presented on forward/side scatter plot for primary culture (A) and after 4 passages (B). The scatter plot was set on a linear scale to indicate particle size and was used to exclude cellular debris and aggregates. The CD166-fluorescein isothiocyanate/CD105-phycoerythrin staining was detected in the primary culture (C) and after 4 passages (D) in geometrical mean fluorescence.

Figure 3: Morphological characterization of chondrocytes (A-C) and CD166+/CD105+ mesenchymal progenitor cells (D-F) from fetal, adult, and elderly patients, respectively, after 2 passages in culture.

Figure 4: Cell growth (1 passage in culture) was measured for mesenchymal progenitor cells from different age groups, indicating a higher grown rate for fetal mesenchymal progenitor cells (A). Cell doubling time was calculated for mesenchymal progenitor cells from different groups of patients, showing a lowest double time (Kruskal-Wallis test, P=.001) (B).
DISCUSSION

Chondrocytes are thought to be terminal cells with low capacity for reproduction or self-renewal. Blood circulation in articular cartilage is also poor, potentially resulting in limited supply of progenitor cells critical for tissue renewal. These factors contribute in part to a high risk for age-related disease in articular cartilage. However, recent studies have found that osteoarthritis chondrocyte express stem cell markers. A key question is whether these mesenchymal progenitor cells undergo functional changes that result in progressively reduced capacity for self-renewal and differentiation. We have provided experimental evidence that human cartilage of all ages contained CD105+/CD166+ mesenchymal progenitor cells in the resting state differentiated into chondrocytes and upregulated the expression of aggregan and type II collagen mRNA in the presence of growth factors.

Osteogenic Differentiation

Levels of alkaline phosphatase in mesenchymal progenitor cells from all age groups increased after osteogenic induction, indicating that cartilage mesenchymal progenitor cells from all ages maintained a normal osteogenic differentiation capacity. However, a 2-week induction resulted in a higher level of alkaline phosphatase in mesenchymal progenitor cells from adult patients as compared to those from fetal and elderly patients, but the difference did not reach statistical significance (Figure 6). Without induction, alkaline phosphatase of mesenchymal progenitor cells from elderly patients was significantly higher than that of the other 2 groups ($P=0.007$), suggesting that mesenchymal progenitor cells from elderly patients’ cartilage might have undergone spontaneous osteogenic differentiation.

Despite their universal presence, we found that fetal mesenchymal progenitor cells had a higher rate of proliferation and chondrogenic capacity as compared to aged cartilage (Figures 4, 5), similar to those found in bone marrow mesenchymal progenitor cells. This low rate of proliferation and slow induction is consistent with finding that aged cartilage had a lower percentage of mesenchymal progenitor cells that had a reduced chondrogenic capacity, as shown in Figure 5. A role of aging in mesenchymal stem cell differentiation has been debated. Our observations strongly suggest a mechanism that supports for age-related development of cartilage degeneration and osteoarthritis.

Furthermore, spontaneous osteogenic differentiation in mesenchymal progenitor cells derived from elderly patients may be related to primary osteoarthritis. For example, mesenchymal progenitor cells cultured in a transforming growth factor-β-containing chondrogenic medium display signs consistent with chondrocyte hypertrophy. Osteoarthritis chondrocytes in culture show significant hypertrophy after transforming growth factor-β induction, which precedes cartilage apoptosis, vessel invasion, and calcification during cartilage development. Xiao et al compared the gene-expression pattern in the bone marrow-mesenchymal stem cells of geriatric (>2 years), osteoporotic and nonosteo-
porotic adult (7 months), and juvenile (7 weeks) rats and detected the highest high expression of osteoblast-related genes in geriatric rats. Together, these findings imply that mesenchymal progenitor cells from elderly animals could promote the development of osteoarthritis by differentiating into bone cells.

Although we have observed significant differences in mesenchymal progenitor cell function in patients from different age groups, the sample size for this study is too small to establish a linear relationship between age and mesenchymal progenitor cell functionalities, due to difficulties obtaining cartilage tissue. It may be more informative to study cartilage mesenchymal progenitor cells at epiphyses fusion age, when cartilage evolves from the growth phase to the stationary phase. This concern is partially addressed by examining fetal cartilage, which represents the growth phase, as well as adult and elderly cartilage, which represent the stationary and degenerative phases. Nevertheless, more studies on the subject are called for, including larger randomized controlled trials to study how mesenchymal progenitor cells associate with osteoarthritis development and to explore the potential of using mesenchymal progenitor cells as a therapeutic alternative to the standard care of patients with osteoarthritis.

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