Assessment of chondrogenic differentiation potential of autologous activated peripheral blood stem cells on human early osteoarthritic cancellous tibial bone scaffold

T. Turajane · T. Thitiset · S. Honsawek · U. Chaveewanakorn · J. Aojanepong · K. I. Papadopoulos

Received: 24 July 2013 / Accepted: 17 October 2013 © Istituto Ortopedico Rizzoli 2013

Abstract

Introduction Current therapeutic regimens in osteoarthritis (OA) address mainly pain but not the slow progressive degradation of the extracellular matrix (ECM) and the loss of a chondrogenic phenotype in articular cartilage. In the present study, using an early OA cancellous bone scaffold, we aimed to uncover evidence of the successful hyaline cartilage regenerative capacity of autologous human granulocyte colony-stimulating factor (hG-CSF)-activated peripheral blood stem cells (AAPBSC) with growth factor addition.

Materials and Methods AAPBSC were harvested in ten patients (median age 58 years, 8 females), and flow cytometry was performed for cell surface markers. Arthroscopically obtained cancellous bone scaffold specimens were seeded with AAPBSC. In Group 1, the scaffold was seeded with AAPBSC only, in Group 2, AAPBSC plus hyaluronic acid (HA), and in Group 3, AAPBSC plus HA, hG-CSF, and double-centrifuged platelet-rich plasma (PRP). The specimens were analyzed for cell attachment and proliferation by the fluorometric quantification of cellular DNA assay and scanning electron microscopy. Chondrogenic gene expression was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) of Sox9, collagen type II (COL-2), and aggrecan. Histological sections of scaffold constructs for cartilaginous matrix formation were stained with toluidine blue (proteoglycan) and safranin O (sGAG) after 3 weeks.

Results AAPBSC displayed especially high levels of CD29 and CD44 surface markers, as well as CD90, and CD105, while only a small proportion expressed CD34. Almost half of the seeded cells attached on the bone scaffolds in all three groups (not statistically significant), whereas the means of cell proliferation on day 7 compared to day 1 were statistically significant difference with the order of increase as group 3 > group 2 > group 1. RT-PCR showed statistically significant sequential increases in Sox9, COL-2, and Aggrecan all being highest in group 3. Histological analysis demonstrated cells in the cancellous bone scaffold with a round morphology, and ECM was positively stained by toluidine blue and safranin O indicating increased proteoglycan and glycosaminoglycan content, respectively, in the newly formed cartilage matrix.

Conclusions AAPBSC initiated chondrocyte differentiation on an autologous cancellous bone scaffold, and the addition of PRP and hG-CSF further stimulated cell proliferation toward a chondrocyte phenotype with potentiated Sox9 transcription resulting in sequential COL-2 and aggrecan mRNA increases that ultimately resulted in histologically confirmed increased proteoglycan and glycosaminoglycan content in newly formed hyaline cartilage.

Keywords Autologous · Peripheral blood · Stem cells · Osteoarthritis · Knee · Granulocyte colony-stimulating factor · hG-CSF · Chondrogenic differentiation · Platelet-rich plasma · Arthroscopy
Background

Articular hyaline cartilage is a specialized, low-friction articular surface tissue in weight-bearing, diarthrodial joints with its main function to absorb, cushion, and protect the underlying bone from forces generated while a joint is being used. Its avascular, alymphatic nature complicates and often gravely impedes its capacity for regeneration and self-healing [25]. Trauma or osteoarthritis (OA), both with a high prevalence and economic impact, creates full-thickness chondral defects that cannot efficiently heal, thus leading to significant long-term disability. When conservative approaches have failed, available invasive treatments, like arthroscopic microfracture, autologous chondrocyte implantation, high tibial osteotomy, or unilateral knee arthroplasty, are generally limited in their ability to regenerate functional cartilage [21]. Recently, successful and encouraging clinical and laboratory results using the combination of intra-articular (IA) autologous peripheral blood stem cells with or without growth factor addition along with hyaluronic acid (HA) in conjunction with arthroscopic microdrilling mesenchymal cell stimulation have been demonstrated in humans [6, 18, 19, 24] and animal models [2, 20] in regenerating articular cartilage in early osteoarthritic knee disease [22].

In this study, the aim was to replicate in vitro the articular environment on early osteoarthritic cancellous tibial bone scaffold in order to uncover corroborative evidence of the successful hyaline cartilage regenerative capacity of our novel preparation of autologous activated peripheral blood stem cells (AAPBSC) with growth factor addition (GFA).

Materials and methods

Between April 2011 and December 2011, ten consecutive patients (median age 58 years, range 56–60 years, eight females) were recruited for the present study. Half of the patients were Kellgren–Lawrence Grade 2, while the remainder were in Grade 3. ICRS grade 3 was seen in four patients, while six were ICRS grade 4. All patients had previously received conventional NSAIDs, but no intra-articular corticosteroid injections in the knee. No systemic or metabolic diseases were noted. The indication for inclusion in the study was failed pharmacological and non-pharmacological conservative treatment in early knee osteoarthritis with quality of life and visual analog scales scores of more than 40 in patients below the age of 60 years. The study protocol was approved by the Hospital’s Ethics Committee and the National Medical Council. All patients signed informed consent forms after having received adequate information on the peripheral blood stem cell collection and discussed the procedure with the treating surgeon.

Procedure outline

The protocol comprised three steps; at first, autologous activated peripheral blood stem cells (AAPBSC) were harvested, and growth factor addition (GFA) concentrate was prepared from autologous platelet-rich plasma (PRP) and was mixed with hG-CSF; then, on the following day, 4–6 specimens of cancellous bone scaffold specimens were obtained (diameter 2 mm, length 6 mm) through needle biopsy in the cancellous bone of the medial femoral condyle during the planned arthroscopy for modified arthroscopic MCS technique with intra-operative injection of the above fresh solution. Normally in arthroscopic surgery, debridement and MCS (mesenchymal stimulation by drilling 2 × 6 mm holes, 3–4 mm apart) take place but in the current study, instead of drilling, needle biopsy was performed obtaining 4–6 specimens per above. The cancellous tibial bone scaffolds were then cut at a diameter of 2 and 4 mm thickness.

To determine the chondrogenic differentiation potential of AAPBSC seeded on human cancellous bone scaffold, our samples obtained from the ten patients were divided into three groups according to three different osteoarthritis treatments protocols. Group 1, with autologous cancellous bone scaffold and AAPBSC, was used as the control reference group, while Group 2 was similar to Group 1 with the addition of 0.1 mg/ml hyaluronic acid (HA), and finally, Group 3, called growth factor addiction preservation (GFA) group, was similar to Group 2 but 0.1 mg/ml hG-CSF and 10 % (v/v) double-centrifuged platelet-rich plasma (PRP) were added. Multiple bone samples were obtained from each patient, and each experiment (Group 1, 2, 3) was conducted thrice on each patient’s samples. The data presented are representative of the three independent experiments.

Attachment and proliferation assays

AAPBSC were seeded on the sterilized cancellous bone scaffolds at 5 × 10^5 cells/scaffold for attachment and proliferation tests using agitation-seeding technique at 200 rpm. The cell-seeded scaffolds were transferred to a tissue culture plate containing proliferating medium Dulbecco’s Modified Eagle Medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10 % fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and 100 U/ml penicillin/streptomycin and cultured at 37 °C in a 5 % CO2 for the attachment and proliferation tests. The medium was refreshed every 2–3 days. The number of cells was analyzed by the fluorometric quantification of cellular DNA according to the method reported by Takahashi et al. [23].
Briefly, the cell samples were lysed in sodium citrate-buffered saline solution (pH 7.4) containing sodium dodecylsulfate at 37 °C overnight. Subsequently, 100 µl of cell lysate was mixed with a fluorescent dye solution (Hoechst 33258 dye) in a 96-well black plate. The fluorescence intensities of the mixed solutions were spontaneously measured at the excitation and emission wavelengths of 355 and 460 nm, respectively. The standard curve between the DNA and cell number was prepared using cells of known numbers.

Chondrogenic induction of hG-CSF AAPBSC

The cancellous bone scaffold constructs seeded with AAPBSC were cultured in T25 flasks at a cell density of 5 × 10^4 cells/cm² in a chondrogenic induction medium containing Ham’s F12:DMEM (1:1) supplemented with 1 % fetal bovine serum (FBS), 1 % antibiotic and antimycotic, 1 % glutamax, 1 % vitamin C, 1 % insulin-transferrin-selenium-X (ITS), 100 nM dexamethasone, 40 mg/mL L-proline, and 5 ng/ml growth factor 1 (IGF-1), 50 mg/mL ascorbate-2-phosphate, 1 % fetal bovine serum (FBS), 1 % antibiotic and anti-mycotic, 1 % glutamax, 1 % vitamin C, 1 % insulin-transferrin-selenium-X (ITS), 100 nM dexamethasone, 40 mg/mL L-proline, and 5 ng/ml transforming growth factor-b3 (TGF-b3) (Sigma Chemical Co., St. Louis, USA) [9]. The morphological features of cultured cells were monitored every day using an inverted light microscope (Olympus, Tokyo, Japan). AAPBSC were harvested at day 7, 14, and 21 of culture for the quantification of sequential chondrogenic gene expression (Sox9, collagen type II, and aggrecan).

Flow cytometry analysis

Flow cytometry was performed on AAPBSC incubated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated antibodies for 30 min. The following CD surface markers were tested: CD34, CD29, CD44, CD45, CD90, and CD105 (BD Biosciences, Franklin Lakes, NJ, USA). Ten thousand events were acquired for each CD surface marker on a Becton–Dickinson FACSCalibur flow cytometer. The data analysis was performed using CELLQuestPro acquisition software (BD Biosciences, Franklin Lakes, NJ, USA).

RT-PCR analysis

For RNA extraction, the cell-seeded cancellous bone scaffold constructs were washed twice with ice-cold PBS, and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Equal amounts of total cellular RNA (5 µg) were reverse-transcribed to cDNA at 42 °C for 60 min in a volume of 20 µl containing the following reagents: 0.5 mM dNTP mix; 1× ImProm-II reaction buffer; 6 mM MgCl₂; and 20 U of recombinant RNasin ribonuclease inhibitor (Promega, Madison, WI, USA). The reactions were then terminated at 70 °C for 15 min. Aliquots of the cDNA were amplified in 100 µl of a PCR mixture which contained 1 µM primer sets, 1× thermophilic polymerase reaction buffer (BioRad), and 5 U of iTaq DNA polymerase (BioRad). Using a thermocycler, DNA amplification was performed including an initial denaturation at 94 °C for 2 min, followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 2 min. The final cycle included 5 min for extension. Glyceraldehyde phosphate dehydrogenase (GAPDH) primers were added in all reactions as an internal control. Furthermore, cDNAs were also amplified using specific primers by RT-PCR to generate products corresponding to mRNA encoding for Sox9, collagen type II, and aggrecan. After amplification, the PCR products were resolved on 2 % agarose gels and visualized with ethidium bromide. The density of each band was quantified using the NIH Image software. Relative gene expression was determined by dividing the densitometric value of treatment by that of the control.

Scanning electron microscopy

The specimens were fixed in 2.5 % glutaraldehyde (pH 7.4) overnight at 4 °C. They were then dehydrated in increasing concentrations of ethanol (from 50, 75, 90 to 100 %) followed by vacuum drying. Dry scaffolds were sputter-coated with gold at 40 mA prior to observing under scanning electron microscope. Scaffold morphology was visualized through scanning electron microscopy (SEM, Joel JSM 5400). To observe the microstructure of the scaffolds, the scaffolds were cut horizontally with a razor blade. The cut scaffolds were placed on the copper mount and sputter-coated with gold (Ion sputtering device, JFC 1100) prior to SEM observation at an accelerating voltage of 12–15 kV.

Histology

The constructs were fixed in 10 % buffered formalin and dehydrated by treatment with a series of graded alcohol and cleared in xylene prior to embedding in paraffin. Samples were cut into 5–10 mm of thickness, deparaffinized, and rehydrated. Hematoxylin and eosin (H&E) staining of sections was done for evaluation of cell morphology in the constructs. Sulfated glycosaminoglycans (GAGs) and proteoglycans were visualized by staining with safranin O and toluidine blue, respectively.

Statistical analysis

Student’s t test was performed to compare the means of two independent groups, and one-way analysis of variance
(ANOVA) was used to compare the means of more than two independent groups. The data are presented as the mean ± the standard error of the mean (SEM). A $p$ value <0.05 was considered to be significant. Statistical analysis was performed using the statistical package for social sciences (SPSS) software, version 16.0 for Windows.

**Results**

AAPBSC harvesting was uneventful and without any side effects prior to (period of 5 day hG-CSF stimulation), during or after the harvesting. Apart from mild swelling and discomfort, the arthroscopies and cancellous tibial bone biopsies were without incidents and no other complaints were noted. In total, ten patients were biopsied, and early osteoarthritic cancellous tibial bone scaffolds were obtained and seeded with human granulocyte colony-stimulating factor (hG-CSF)-activated autologous peripheral blood stem cells (AAPBSC).

On flow cytometry analysis, AAPBSC displayed positive staining for the mesenchymal surface markers CD29, CD44, CD90, and CD105 and exhibited especially high levels of expression, staining more than 80 % of the total cell population, of CD29 (84.8 %) and CD44 (94.83 %), while CD90 (4.56 %) and CD105 (8.84 %) were seen in lower numbers. In contrast, only a small proportion of AAPBSC expressed the hematopoietic markers CD34 (0.05 %).

Almost half of the seeded cells attached on the bone scaffolds in all three groups and the differences of cell attachment were not statistically significant, whereas the means of cell proliferation on day 7 showed statistically significant differences with the order of increase as group 3 (AAPBSC + HA + GFA) > group 2 (AAPBSC + HA) > group 1 (AAPBSC only); group 3 vs. group 1, $p < 0.001$; group 3 vs. group 2, $p < 0.03$ (Fig. 1).

On visual inspection of scanning electron microscopy pictures, we noticed increase in cell attachment and cell proliferation in group 3 compared to group 2 and group 1 (Fig. 2).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was performed on chondrogenic differentiation markers such as Sox9, collagen type II (COL-2), and aggrecan. Sox9 increased in day 7 and 14, and the order of increase was group 3 (AAPBSC + HA + GFA) > group 2 (AAPBSC + HA) > group 1 (AAPBSC only) at both occasions and statistically significant on day 14 ($p < 0.05$), planning out on day 21 while COL-2 significantly increased throughout to day 21 (group 3 > group 2 > group 1) ($p < 0.001$). Aggrecan increases were statistically significant on days 7 and 14 ($p < 0.01$) and leveled out on day 21 (Fig. 3).

Histological analysis demonstrated that the cells in the cancellous scaffold had a round morphology, and the extracellular matrices were positively stained by safranin O and toluidine blue (Fig. 4). Safranin O staining indicates glycosaminoglycan, and toluidine blue allows assessing metachromatic staining of cartilage matrix due to proteoglycan content witnessing for increased content of both glycosaminoglycan and proteoglycan production, the newly formed cartilage matrix.

**Discussion**

Osteoarthritis (OA) is characterized by the slow progressive degradation of the extracellular matrix (ECM) and the
Fig. 2  Scanning electron micrographs of cancellous bone scaffold seeded AAPBSC. Scanning electron micrographs of cancellous bone scaffold (A) and autologous human granulocyte colony-stimulating factor (hG-CSF)-activated peripheral blood stem cells (AAPBSC) on cancellous bone scaffold on days 1 (d1) and 7 (d7) for B = AAPBSC + cancellous bone scaffold as control on d1, E = AAPBSC + cancellous bone scaffold as control on d7, C = AAPBSC + cancellous bone scaffold + hyaluronic acid on d1, F = AAPBSC + cancellous bone scaffold + hyaluronic acid on d7, D = AAPBSC + cancellous bone scaffold + hyaluronic acid + hG-CSF + platelet-rich plasma on d1, G = AAPBSC + cancellous bone scaffold + hyaluronic acid + hG-CSF + platelet-rich plasma on d7. Data are representative of three independent experiments.
loss of a chondrogenic phenotype in articular cartilage [25]. Current therapeutic regimens address mainly pain but not degeneration. Strategic targeting of therapeutic genes to OA cartilage may offer potent alternatives for restoring the structure of the damaged cartilage [1]. The avascular and alymphatic nature of the articular cartilage necessitates a scaffold that will successfully bring in the necessary nutrients and hold the operative cells in place to exert their effect whether that is fusion with resident cells, stimulation and differentiation of local chondrocyte precursors, paracrine support, or a combination of all of the above [22, 24]. Recently, it was demonstrated that the combination of intra-articular (IA) autologous activated peripheral blood stem cells (AAPBSC) with or without growth factor addition (GFA) along with hyaluronic acid (HA) in conjunction with arthroscopic microdrilling mesenchymal cell stimulation (MCS) resulted in quality of life improvements measured by WOMAC and KOO scores and succeeded in
regenerating articular cartilage in early osteoarthritic knee disease that failed conservative treatment and versus non-cellular interventions [19, 24]. Similarly, encouraging 3-year results have been reported by Buda et al. [3] in osteochondral lesions of the knee using autologous bone marrow stem cells harvested from the patient’s posterior iliac crest.

In the present study, we evaluated the in vitro mechanisms possibly underlying the observed clinical improvement. Cartilage is composed of cells named chondrocytes and the extracellular matrix (ECM) produced by these cells. The biochemical properties of cartilage and the physical function of joints are critically dependent on the integrity of the matrix. The ECM molecules in cartilage include proteoglycans, hyaluronan (also called hyaluronic acid or HA), type II collagen, glycoproteins, and various mixtures of elastic fibers. Most of the proteoglycans exist as aggregates formed by the non-covalent association of proteoglycan with HA and link protein, and the most crucial to the proper functioning of articular cartilage is aggregan, one of the large aggregating chondroitin sulfate proteoglycans. Along with type 2 collagen, aggregan forms a major structural component of cartilage, particularly articular cartilage. Moreover, aggregan plays an important role in mediating chondrocyte–chondrocyte and chondrocyte–matrix interactions through its ability to bind hyaluronan [10].

Autologous activated peripheral blood stem cells (AAPBSC), collected from the patients, were seen to contain satisfactory numbers of CD105 cells thought to be both endothelial progenitor cells (EPC), aiding angiogenesis, and mesenchymal stem cells (MSC) with chondrocyte precursor potential to initiate hyaline cartilage remodeling [5, 26]. In our study, flow cytometry analysis of AAPBSC displayed positive staining for the mesenchymal surface markers CD29, CD44, CD90, and CD105 and exhibited especially high levels of expression of CD29 and CD44, which stained more than 80% of the total cell population. Thus, these findings indicated that our cell preparation contained a sufficient mixture of EPC and MSC to initiate resurfacing. EPC may be of importance in ensuring an adequate balance between availability and handling of oxygen in developing growth cartilage to preserve chondrocyte survival [12]. The regenerative potential of autologous platelet-rich plasma (PRP) is based on the release of growth factors that occurs with platelet rupture and its addition may deliver crucial growth factors such as transforming growth factor (TGF) beta, insulin-like growth factor 1 (IGF-1), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), bone morphogenetic proteins (BMP), and platelet-derived growth factor (PDGF), all known to promote stimulation of blood vessel, chondrocyte, and ECM formation [11, 15, 27] were also available in our preparation. Recently, chondrogenic markers (Sox9 and aggregan) have been confirmed to increase following PRP administration suggesting that PRP may enhance MSC proliferation and cause chondrogenic differentiation of MSC in vitro [13].

In electron microscopy, we noted significantly increased AAPBSC proliferation both qualitatively and quantitatively between day 14 and day 7, the increase being highest in the GFA group 3. Human genetic approaches have revealed
that Sry-type high-mobility group box gene, Sox9, plays a paramount role in chondrocyte fate and differentiation and has been identified as the master transcription factor in chondrogenesis, activating the transcription of target genes as collagen type II (COL-2) and aggrecan in a multistep function [7]. We noted temporal and sequential increases of Sox9 peaking on day 14 and being highest in the GFA group, while COL-2 increased on day 14 and 21 also being highest in the GFA group. Aggrecan increases were significantly different between the groups, similarly being highest in the GFA group on day 14 and further increased until day 21.

The addition of intraarticular hG-CSF may further enhance cartilage regeneration by attracting additional bone marrow stem cells, aiding angiogenesis in an appropriate environment, and regulating the proliferation, migration, or differentiation of the adult mesenchymal stem cells [17] locally. Moreover, the advantageous effects of hG-CSF in bone tendon integration may extend to the subchondral area [17]. Interestingly, GM-CSF also strongly stimulates proteoglycan and collagen synthesis, and this observation may offer an additional explanation to the effects observed in our model [16]. Furthermore, hG-CSF has recently been shown to attenuate the impact of aging on bone marrow stem cells and recover age-related functional decline by significantly improving their proliferation activity and growth factor production such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). Both of the latter are found in the synovial membrane, expressed in articular chondrocytes, and play a role in knee arthritis [8], thus bypassing age-related limitations and objections in using autologous peripheral blood stem cells in cartilage repair [4]. Moreover, recent evidence suggests that hematopoietic stem cells are pluripotent and may be the source for the majority, if not all, of the cell types in our body, thus overcoming the need for other sources of stem cells that lack the safety precedence of more than a century of use of hematopoietic stem cells [14].

Thus, on a molecular and cellular level, the seeded AAPBSC, aided by GFA, attached and significantly proliferated, the Sox9 master transcription factor increased, sequentially cascading COL-2 and aggrecan increases, indicating viable chondrocyte units producing ECM. The hyaline nature of the ECM was confirmed by histological studies with toluidine blue staining and safranin O staining indicating proteoglycan and glycosaminoglycan content.

**Conclusion**

In the present study, we report corroborative evidence for the hypothesis that autologous hG-CSF-activated peripheral blood stem cells (AAPBSC) contain satisfactory numbers of EPC and MSC to initiate chondrocyte differentiation on an autologous cancellous bone scaffold and the addition of PRP and hG-CSF further stimulated cell proliferation toward chondrocyte phenotype with potentiated Sox9 transcription resulting in sequentially elevated COL-2 and aggrecan expression that ultimately resulted in histologically increased proteoglycan and glucosaminoglycan content in newly formed hyaline cartilage.

**Acknowledgments** The authors would like to thank the Research Core Facility of the Department of Biochemistry and Chulalongkorn Medical Research Center (ChulaMRC) for kindly providing facilities; Dusit Vichakorn Foundation, Surgeon in Chief Foundation, Police General Hospital, TRB Chemidica Co., Ltd.; Dr. Juthatip Fongsarun for performing the leukaphereses and all patients and their families for participating in the study.

**Conflict of interest** THAI StemLife Co., Ltd. has financially supported the autologous leukaphereses in the 10 samples tested. KP holds shares in THAI StemLife’s founding company and is an executive board member of THAI StemLife. The remaining authors have no competing financial or non-financial interests.

**References**


