Amniotic Fluid-derived MSC Secretome Halts Action of IL-1 β and TNF- α Through ERK/MAPK and Returns Cartilage Repair Under OA Inflammatory Stimuli

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Research Article

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Abstract

Background: Osteoarthritis (OA) is a degenerative cartilage disease. OA cartilage has a limited repair capacity due to the effect of IL-1 β and TNF- α on the chondrocyte progenitor cells (CPC) in an OA joint. Mesenchymal stem cells (MSC) therapy is a therapeutic option for osteoarthritis that initiated by the ability of secretory growth factors and mediator molecules to heal OA. Amniotic fluid MSC (AF-MSC), an interesting MSC source, has been shown to secrete various growth factors and anti-inflammatory molecules promoting tissue repair and regeneration. However, the effect of AF-MSC secretory factors to inflammation and cartilage repair is still limited. The current study aims to explore the action of AF-MSC secretome to IL-1 β and TNF- α , and the CPC function that encourages cartilage repair.

Methods: The effect of AF-MSC secretome to OA inflammatory cytokines was observed via the CPC migration using scratch assay. Inhibitory action of AF-MSC secretome to IL-1 β and TNF- α was determined through NF- κ B and MAPK signaling pathways by western blot. The repaired function of OA cartilage was analyzed via the cartilage outgrowth study and the expression of chondrogenic and anabolic genes using qRT-PCR.

Results: AF-MSC secretome can arrest inflammatory action of IL-1 β and TNF- α and reduces production of NF- κ B, pNF- κ B, p38, pp38, ERK, COX-2, and iNOS signaling proteins. It significantly reduced the production of pERK ($P = 0.0434$). For cartilage repair, AF-MSC secretome promotes CPC outgrowth and migration in human OA cartilage, even under inflammatory stimuli. By the action of AF-MSC secretome, the inflamed CPC can restore *Col II* and anabolic genes; *IGF1* expression, indicating reactivation of cartilage regeneration.

Conclusion: AF-MSC secretory factors have the ability to halt inflammatory actions of IL-1 β and TNF- α via the ERK/MAPK pathway and motivate CPC function and anabolic property.

Background

Osteoarthritis (OA) is a chronic progressive disease of articular cartilage. The pathophysiological process in an OA knee was mediated by inflammatory cytokines produced by synovium and chondrocytes [1]. The distinctive cytokines accumulated in the knee synovial fluids of OA patients are interleukin-1 β (IL-1 β) and tumor necrosis factor α (TNF- α) [2]. IL-1 β and TNF- α down-regulate an anabolic action and up-regulate a catabolic action of cartilage, resulting in cartilage degeneration and limiting intrinsic self-repair of endogenous chondrocyte progenitor cells (CPC) [3, 4]. IL-1 β and TNF- α induce the inflammatory responses through the activation of the MAP kinases, NF- κ B, and the encoding of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) [5].

Mesenchymal stem cells (MSC) therapy has shown to be a promising alternative treatment for incurable and degenerative diseases. MSC transplantation is a therapeutic option for OA. A primary benefit of MSC transplantation is its paracrine effect, not its ability to directly differentiate into chondrocyte to repair tissue [6]. During exogenous MSC implantation, MSC secrete anti-inflammatory trophic factors,

chemokines, and growth factors into the damaged area of the articular cavity. The paracrine trophic factor will affect the downregulation of tissue inflammation, inhibit chondrocyte apoptosis, and also promote endogenous chondrocyte progenitor cells proliferation resulting in cartilage regeneration [7, 8]. It is believed that MSC secretome enriches with enzymes to restore extracellular matrix balance and is effective for OA treatment [9]. MSC secretome is primed to become the main option for clinical OA treatment.

MSC for therapeutic application can be provided from various tissue sources, which present similar major MSC characteristics. However, MSCs derived from different tissue origins can present their own unique characters. The strategy of exogenous stem cell therapy is using stem cell types that are suitable for precise treatment. Amniotic fluid-derived MSC (AF-MSC) is obtained from fetal tissue that originates through routine amniocentesis with minimal invasive procedure. AF-MSC has distinguishing characteristics with a high potential for multi-lineage differentiation, cell proliferation, genomic stability, presenting of HLA-G, secreting various growth factors, and anti-inflammatory cytokines, but is not tumorigenic [10, 11]. AF-MSC represents a valuable source for cell-based therapy in degenerative and inflammatory diseases [12]. A recent study demonstrated that AF-MSC can secrete immunoregulatory factors that are useful in treating autoimmune disease and allogeneic implantation [13]. Moreover, AF-MSC uniquely expresses interleukin-4 (IL-4), rarely expressed in other MSC types [13, 14]. IL-4 has a potential to downregulate the action of IL-1 β and TNF- α and exert a remarkable pro-survival and anti-apoptotic effect to promote tissue repair [15, 16].

To explore the potential of AF-MSC for OA therapy, the current study observed the role of AF-MSC secretome to OA from the anti-inflammation and tissue repair effects. Using scratch assay, we investigated an action of AF-MSC secretome to major OA inflammatory cytokines; IL-1 β and TNF- α . We also investigated the action of inflammatory signaling proteins in nuclear factor- κ B (NF- κ B) pathway and mitogen-activated protein kinase (MAPK) signaling pathway using western blot. In tissue repair, we observed endogenous CPC function via outgrowing cells from OA cartilage and determined anabolic gene action on CPC using real-time RT-PCR.

Methods

Human materials

Cartilage was provided from knee biopsies of seven osteoarthritis patients (age over 60 years), who underwent total knee replacement at the Department of Orthopedic Surgery, Faculty of Medicine, Siriraj Hospital. The participants gave informed consent and signed a document to allow the use of specimens for the investigation. The consent document was approved by the Ethics Committee of Siriraj Hospital (Si 075/2014), Mahidol University, Thailand.

Culture medium

The culture medium used in the experiment can be divided into three types. Control medium is chondrocyte culture medium (CC medium), containing Dulbecco's Modified Eagle Medium (DMEM; Gibco,

Invitrogen, CA) with a supplement of 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO). The inflammatory medium is CC medium supplemented with IL-1 β /TNF- α combination. The secretome medium is inflammatory medium supplemented with 50% v/v AF-MSC secretome.

Culture and establishment of chondrocyte progenitor cells (CPC) from cartilage

Cartilage tissues in healthy non-weight-bearing area, which were not presented as an OA lesion, were selected. The cartilages were cut from macroscopically intact and non-fibrillated regions into small pieces about 3 $\times$ 3 mm. The cartilage slices were placed in a 60 mm tissue culture dish under chondrocyte culture medium (CC medium) at 37°C, 5% CO₂. Medium was changed every four days. Cartilage slices were removed after CPC began growing from cartilage tissue. The outgrowing cell was scaled up by repetitive subculture passaging and used for study.

Preparation of AF-MSC secretome

AF-MSC secretome were supported by Department of Obstetrics and Gynecology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. Preparation of secretome was performed by cell seeding at a density of 7,000 cells/cm² into a T75 tissue culture flask and incubated under alpha minimum essential medium (α -MEM) supplemented with 10% of ES-FBS and 1% penicillin-streptomycin until they reached 70% confluence. The medium was replaced with 5 ml α -MEM with a serum-free for 24 h at 37°C, 5% CO₂. The culture medium was collected and filtered through a 0.22 μ m filter membrane and designated as AF-MSC secretome and stored at -80°C for further use.

Scratch assay for cell migration

To provide appropriate concentration of inflammatory cytokine for the experiment, we optimized concentrations of IL-1 β and TNF- α based on the ability to inhibit CPC migration. Scratch assay was performed under CC medium supplemented with gradient concentrations of IL-1 β (ImmunoTools, Friesoythe, Germany) at 0.02, 0.2, 1 ng/ml, and TNF- α (ImmunoTools) at 0.05, 0.5, 5, and 10 ng/ml. The optimal concentrations of IL-1 β and TNF- α were used to perform a combination of IL-1 β and TNF- α (IL-1 β /TNF- α combination) and applied for use in all experiments of this study.

To investigate the effects of AF-MSC secretome to IL-1 β /TNF- α combination, a scratch assay was performed under three different media, CC medium as a control, inflammatory medium, and secretome medium.

CPC were plated at an initial density of 1.5 $\times$ 10⁴ cells/cm² in a 35 mm tissue culture dish under CC medium. When a confluent monolayer was formed, the CPC was scraped to make 0.5 cm gap distance for a migration area. A scratching line was created on the migration area.

The cultures were incubated for seven days with a daily medium change. The number of cells that migrated past the scratching lines was observed and compared at day one, three, five, and seven using

phase contrast inverted microscopy. The experiment was repeated five times using different CPC samples.

Cytoplasmic and nuclear proteins extraction

CPC was grown until it reached 70% confluence and divided into three groups to incubate in three different medium conditions. CPC in Group 1 was incubated under CC medium, whereas, CPC in Group 2 and 3 were incubated under basal medium supplemented with IL-1 β /TNF- α combination for 3 h (inflamed CPC) before subsequently shifted to incubate under either CC medium (Group 2), or 50% v/v AF-MSC secretome medium (Group 3) for 24 h. After culture, cells were extracted for cytoplasmic and nuclear proteins. For protein extraction, CPC was scraped from tissue culture dish and washed with cold PBS before re-suspending in hypotonic buffer by pipetting and incubating 15 min on ice. The cell membrane was lysed by adding a 10% NP-40 (Abcam, Cambridge, UK) before vortexing and centrifugation. The supernate was collected for cytoplasmic protein. The pellet was further lysed by vortexing in ice-cold nuclear extraction buffer. Chromatin was removed by centrifugation. The supernate was collected for soluble nuclear protein.

Western blot analysis

Ten micrograms of proteins were subjected to 10% SDS-PAGE and transferred onto PVDF membranes (Bio-Rad, Hercules, CA). The membranes were washed and a non-specific protein was blocked with 5% skim milk (Himedia, Mumbai, India) in TBST buffer before incubated overnight at 4°C with primary antibodies; iNOS (ab178945), COX-2 (ab62331), NF- κ B p65 (ab32536), pNF- κ B p65 (ab76302), p38 (ab170099), pp38 (ab195049), ERK1/ERK2 (ab184699), pERK (ab76299), beta Actin (ab8226) (all from Abcam), and Histone H3 (#3638S, Cell signaling, Denvers, MA). The immunoblot was then washed and incubated with anti-rabbit IgG (HAF008, R&D systems, Minneapolis, MN) or anti-mouse IgG (ab97046, Abcam). The immunoblot was detected using ImageQuant LAS4010 system. The protein band intensities were quantified using the ImageJ program. The levels of proteins in cytoplasmic fraction and nuclear fraction were respectively provided as a relative ratio to beta Actin or Histone H3.

Endogenous cell outgrowing from cartilage tissue

To investigate the effect of AF-MSC trophic factors to cartilage under inflammation that mimicks the condition of an OA knee joint, we observed the action of CPC under the culture supplemented with both inflammatory cytokines and AF-MSC secretome. Cartilage tissue from the area of the OA lesion was dissected into small pieces (3 $\times$ 3 mm) and incubated in a 96 well plate under three different media including, (1) CC medium as control medium, (2) inflammatory medium, and (3) secretome medium. The medium was changed twice a week. CPC outgrowth from cartilage tissues was observed using inverted microscopy. The number of outgrowth cartilage slices and migratory cells in each tissue were recorded until day 21 of culture.

Gene expression analysis

To study the effect of IL-1 β and TNF- α and AF-MSC secretome on molecular change in CPC, the expression of genes that effect articular chondrocyte anabolic activity and chondrogenic genes were analyzed using quantitative real-time RT-PCR (qRT-PCR). Total RNA was extracted from CPC under a control medium, inflammatory medium, and secretome medium for 96 h using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules, CA) and amplified using selective primers (Table 1). The qRT-PCR was performed on LightCycler 480 using SYBR Green I master (Roche Diagnostics GmbH, Mannheim, Germany). The data were analyzed using the $2^{-\Delta\Delta CT}$ method and determined relative to the quantity of *beta Actin*. Experiment was performed in triplicate.

Table 1
Primers of chondrogenic and anabolic activity genes

Gene	Primer sequences (5'-3')	Accession No.	Annealing Temp (°C)	Product size (bp)
<i>beta-Actin</i>	F: 5'-ATGTGGCCGAGGACTTTGATT-3' R: 5'-AGTGGGGTGGCTTTTAGGATG-3'	NM_001101.5	60	107
<i>Col I</i>	F: 5'-AGGACAAGAGGCATGTCTGGTT-3' R: 5'-GGACATCAGGCGCAGGAA-3'	NM_000088.3	57	122
<i>Col II</i>	F: 5'-GGCAATAGCAGGTTTACGTACA-3' R: 5'-CGATAACAGTCTTGCCCCACTT-3'	NM_033150.3	60	79
<i>IGF1</i>	F: 5'-AAGATGCACACCATGTCCTCC-3' R: 5'-AGCCTCCTTAGATCACAGCTCC-3'	NM_001111285.3	58	248
<i>RUNX2</i>	F: 5'-ATGCTTCATTCGCCTCAC-3' R: 5'-ACTGCTTGCAGCCTTAAAT-3'	NM_001024630.3	57	156
<i>SOX9</i>	F: 5'-CCCAACAGATCGCCTACAG-3' R: 5'-TTCTGGTGGTCGGTGTAGTC-3'	NM_000346.4	57	97
<i>TGFβ1</i>	F: 5'-GGGACTATCCACCTGCAAGA-3' R: 5'-CCTCCTTGGCGTAGTAGTCG-3'	NM_000660.7	59	239

Statistics

Data (the mean $\pm$ standard error of the mean; SEM) were analyzed by student's t-test and one-way ANOVA with Tukey post-test using PRISM software version 8.0 (GraphPad Software). Values of $P < 0.05$ were considered to indicate statistically significant differences.

Results

Cells and trophic factors

Explant CPC from cartilage tissues cultured under chondrocyte medium were observed after day three of incubation and scaled up to passage three. The cells were observed in fibroblastic morphology (Fig. 1A) with a population doubling time (PDT) at 2.73 ± 0.38 days and a presence of cell surface markers CD29 (98.4%), CD73 (99.8%), CD90 (99.9%), CD105 (99.9%), CD34 (1.4%), and CD45 (0.9%).

AF-MSC for secretome derivation presented typical MSC characteristics. The cells had a spindle fibroblast-like morphology with a PDT at 1.42 ± 0.21 days and a high presence of cell surface markers CD29 (99.6%), CD44 (99.6%), CD73 (99.9%), CD90 (98.6%), CD105(99.5%), and an *in vitro* multi-lineage differentiation ability. AF-MSC secretome was provided from the culturing of AF-MSC 2×10^6 cells in 5 ml culture medium for 24 h. Total protein concentration in secretome was presented at 0.69 mg/ml.

AF-MSC secretome inhibits effect of IL-1 β and TNF- α

To provide an *in vitro* inflammatory circumstance mimicking an OA knee, an appropriated concentration of IL-1 β and TNF- α was defined and showed that IL-1 β at the concentrations of 0.2 and 1 ng/ml and TNF- α at 0.5, 5, and 10 ng/ml significantly inhibited the migratory action of CPC in scratch assay (Fig. 1B). IL-1 β at a concentration of 1 ng/ml and TNF- α at a concentration of 10 ng/ml were used to provide IL-1 β /TNF- α combination in an inflammatory medium for experiment.

On the contrary, AF-MSC secretome showed a potential to motivate CPC migration even with cells under a fully inflammatory stimulus. In scratch assay, CPC under an inflammatory medium, a secretome medium and control medium can move straight to the migration area within 24 h of culture. No cell in any medium moved across the scratching line. At day three of the experiment, AF-MSC secretome arrested the action of IL-1 β /TNF- α by promoting a migration effect of CPC. The number of migratory CPC found under the secretome medium was a significantly higher amount than the migratory cell under control medium ($P = 0.0121$) and inflammatory medium ($P < 0.0001$) in the migration area (Fig. 1C). At day five of examination, CPC under an inflammatory medium carried typical morphology, but stopped migration and clustered in a limited area, whereas, CPC under secretome medium moved straight along to a free area and showed superiority of cell migration. The amount of CPC in migration area that passed over the scratching line was counted and the amount of migratory CPC under secretome medium was significantly higher than cells under inflammatory medium ($P < 0.0001$) and control medium ($P < 0.0001$) (Fig. 1C). At day seven, CPC under inflammatory medium did not improve cell migration, whereas, migratory CPC under the secretome medium was approximately four times more than in an inflammatory

medium ($P < 0.0001$) and two and one-half times higher than migratory cells in a control medium ($P < 0.0001$) (Fig. 1C). Our work indicated that AF-MSC secretome has a crucial effect to restart the migration property of CPC, even under OA inflammatory stimuli.

AF-MSC secretome inhibits action of IL-1 β and TNF- α via ERK/MAPK pathway

AF-MSC secretome has the potential to arrest the inflammatory action of IL-1 β and TNF- α . To understand the role of AF-MSC secretory factors in the anti-inflammation effect, we investigated by western blot the cascading proteins in signaling pathways of NF- κ B and MAPK in the cytoplasm and nucleus of CPC. CPC was incubated under inflammatory IL-1 β and TNF- α combination to provide the inflamed CPC.

We found that inflamed CPC that was incubated under CC medium presented high levels of signaling proteins in both NF- κ B and MAPK pathways, whereas, inflamed CPC subsequently incubated under AF-MSC secretome expressed a low level of inflammatory signaling proteins in both cytoplasmic and nuclear fractions. In the NF- κ B signaling pathway, NF- κ B and pNF- κ B proteins in both the cytoplasm and nucleus increased in inflamed CPC incubated under CC medium and declined in inflamed CPC incubated in AF-MSC secretome (Fig. 2A).

In the MAPK signaling pathway, AF-MSC secretome has the effect of reducing p38, pp38, ERK, and pERK protein expression in both the cytoplasmic and nucleus. The cytoplasmic p38, pp38, ERK, and pERK were presented at high levels in inflamed CPC under CC medium and in low levels in inflamed CPC incubated under AF-MSC secretome. However, no significant difference in the expression levels of p38, pp38, ERK, and pERK were found among control CPC, and inflamed CPC incubated under either CC medium, or in the AF-MSC secretome. In the nucleus, the expression of p38, pp38, ERK, and pERK exhibited in the same manner to its presence in cytoplasm. It was found at a high level in inflamed CPC under CC medium as compared to control CPC, and inflamed CPC under AF-MSC secretome (Fig. 2B, C). Nuclear pERK was significantly reduced by AF-MSC secretome. A significantly lower expression level of pERK was presented in inflamed CPC incubated under AF-MSC secretome as compared to inflamed CPC incubated under CC medium ($P = 0.04340$) and control CPC ($P = 0.8568$) (Fig. 2C).

For COX-2 and iNOS expression, AF-MSC secretory factors have a role in reducing the expression of COX-2 and iNOS in both the nucleus and cytoplasm. Inflamed CPC incubated in AF-MSC secretome reduced the expression of COX-2 and iNOS, whereas, overexpression of COX-2 and iNOS was found in inflamed CPC incubated in CC medium (Fig. 2D, E). From our results, AF-MSC secretome has the effect of reducing signaling proteins in inflammatory pathway via the ERK/MAPK pathway.

AF-MSC secretome stimulates endogenous CPC growth in OA cartilage

Forty-eight cartilage slices were incubated under control CC medium, inflammatory medium, and secretome medium. At day 10, we observed endogenous CPC outgrowth from cartilage slices under three culture media. The amount of cartilage tissues presenting a CPC outgrowth under secretome medium was comparable to the amount of outgrowth tissues under the control medium, but superior to the

inflammatory medium. At day 14, outgrowth tissues were observed in 11 of 48 (22.9%) tissue slices in control medium, seven of 48 (14.8%) slices in inflammatory medium, and 12 of 48 (25%) slices in secretome medium. CPC morphology was present in fibroblastic cell type (Fig. 3A). The number of outgrowth tissues in inflammatory medium did not increase after day 14. At day 21 of cartilage culture, the amount of outgrowth cartilage tissue in secretome medium was more than the cartilage amount in inflammatory and control medium (Fig. 3B). The outgrowth cells produced under secretome medium migrated into the free culture area, whereas, the outgrowth cells in inflammatory medium migrated a limited distance as a bunched cluster, not found in other mediums. The number of migratory cells in secretome medium was significantly higher than those found in control medium ($P = 0.0341$) and inflammatory medium ($P = 0.0396$) (Fig. 3C).

CPC under control and in secretome medium showed a typical fibroblastic appearance and high proliferation potential, whereas, migratory CPC in the inflammatory medium became enlarged and older. Our work indicates AF-MSC secretome inhibits the action of IL-1 β and TNF- α , and stimulates cartilage regeneration and repair, even under inflammatory circumstance.

Expression of cartilage regeneration genes under adverse stimuli

We then investigated the molecular effect of AF-MSC secretome to chondrogenic genes as well as anabolic genes in CPC under OA inflammatory circumstance. The result showed that AF-MSC secretome activates the expression of cartilage regeneration genes. *Col I*, *RUNX2* and *SOX9* showed no difference in the expression level in CPC from the inflammatory medium and secretome medium. We found that *Col II* expression showed a four times reduction in CPC under inflammatory medium as compared to the control CPC. However, AF-MSC secretome can inhibit altered function of chondrogenic genes. We found that the CPC in secretome medium, composed of both IL-1 β /TNF- α combination and AF-MSC secretome, can return *Col II* expression and showed a comparable expression level to CPC under the control medium (Fig. 4A). For gene involving anabolic action in cartilage, the expression level of *IGF1* in CPC under secretome medium was 300 times more than CPC in control medium, and six times more than CPC in inflammatory medium. For *TGF β 1*, CPC under secretome medium and inflammatory medium showed a comparable expression level, but at two times less than the expression in control (Fig. 4B).

Discussion

Due to the advantage of MSC to differentiate into multi-lineage, *in vitro* scalable, secrete anti-inflammation trophic factors, growth factors, and healing defect tissues, MSC therapy has been considered an alternative treatment for various incurable diseases. Amniotic fluid MSC is an effective MSC source, given its proliferation ability over adult MSC, and as it is provided from fetal tissue during a period of organ development and secretes unique growth factors and cytokines. The current study investigates the potential and biochemical process of AF-MSC secretory factors for OA therapy. The experiment modeled an OA environment with a high level of inflammatory IL-1 β and TNF- α cytokines. The work showed that AF-MSC secretome can arrest the action of IL-1 β and TNF- α and induce a significantly

amount of CPC to migrate. The secretome can stimulate endogenous CPC outgrowth from cartilage tissue and proliferate even under inflammatory stimuli, implying its potentials for cartilage regeneration and repair. The study also investigated the action of AF-MSC secretome to the NF- κ B and MAPK inflammatory signaling pathway and that AF-MSC secretome inhibited the inflammatory action of OA via the ERK1/2 MAPK pathway. AF-MSC secretome appeared to inhibit production of ERK in cytoplasm and also arrested the phosphorylation and translocation of pERK from cytoplasm to nucleus. It resulted in decreased COX-2 and iNOS production (Fig. 5). Our work also demonstrated that AF-MSC secretome can return anabolic action in inflamed CPC and induce cartilage regeneration as seen through high expression of *IGF1* and *Col II* genes and cartilage regeneration genes even under inflammatory stimuli.

Micro-fracturing (MF) and autologous chondrocyte implantation (ACI) were developed from cell-based therapy. MF is a surgical method that drills subchondral bone to recruit bone marrow stem cells to the site of degenerated cartilage to encourage tissue regeneration and repair. ACI is a surgical method to biopsy cartilage in non-weight bearing area to provide endogenous chondrocyte to implant into OA lesion. However, the helpful outcome of cartilage repair in ACI can be effective only in healthy young and active patients, not in OA patients [17, 18] whereas, MF showed low success in long term repair, but was harmful in elderly people [19, 20]. A large amount of clinical follow-up showed that MF and ACI developed fibrocartilage and delayed OA only in the early stage [18, 21]. Currently, exogenous MSC transplantation is an optional treatment for OA. MSC therapy has a major effect in induction of the homing of endogenous stem cells in cartilage. This technique can recruit a repair function of autologous endogenous chondroprogenitor cells and is more effective in forming typical hyaline cartilage than MF and ACI [22–25]. The beneficial role of exogenous MSC to articular cartilage regeneration is mainly supported in two theories. First, it is described as “differentiation theory”, which states that stem cells directly differentiate into chondrocytes and replace damaged chondrocytes in cartilage. Second, it describes “paracrine theory”, in which stem cell secret bioactive trophic factors to trigger biological behavior of chondrocyte and endogenous stem cells [25–27]. Stem cell therapy is benefitted by the paracrine effect of therapeutic delivery agents. However, stem cell differentiation and replacement to OA lesion has proved difficult to achieve owing to the progression of MSC differentiation into inappropriate chondrocyte hypertrophy [25, 27–30] so that only a few cells can survive for a few weeks after transplant [25, 31, 32]. A number of studies have suggested avoiding the injection of cells by using MSC secretome for OA therapy [16, 33]. Our current work supports the conclusion that MSC has therapeutic action through secretory paracrine factors. AF-MSC secretory proteins is a promising tool for OA therapy.

Our work demonstrated that CPC cultured under IL-1 β and TNF- α inflammatory cytokines showed low expression levels of collagen type 2 gene, whereas, CPC under AF-MSC secretome showed up-regulation of collagen type II synthesis. During OA defect, IL-1 β and TNF- α cytokines were derived from chondrocyte and synovial cells. IL-1 β and TNF- α subsequently induced quiescent chondrocyte in OA cartilage to produce matrix-degrading enzymes [3, 18, 34, 35]. MMP acts to cleave collagen and aggrecan promoting ECM degradation and remodeling; for example, MMP-13, a major enzyme to hydrolyze and degrade type II collagen, MMP-3, aggrecanase enzyme, and co-function of MMP-1, MMP-8, and MMP-9 cleaves to the triple helix, and unwinds the collagen chains. MMPs can also co-act with IL-1 β and TNF- α and then

produce subsequent disruption of collagen type II synthesis. Reduction of IL-1 β and TNF- α has been associated with enhancement of collagen type II synthesis. With potential of AF-MSC secretome to arrest inflammatory action of IL-1 β and TNF- α , CPC under AF-MSC secretome medium showed high collagen type II synthesis as compared to CPC under inflammatory medium. Evidence of MSC secretory factor to inhibit inflammation in OA cartilage has been presented. For example, the finding of Simental-Mendia *et al.*, which presented AD-MSC culture medium that can down-regulate the expression of IL-1 β in chondrocyte explanted from OA cartilage [36]. Other evidence by Chen *et al.* revealed BM-MSC derived-trophic factors can down-regulate the expression of IL-1 β , TNF- α , and IL-6 genes in inflamed chondrocytes, which were induced to inflammatory cell with lipopolysaccharide [37].

IL-1 β and TNF- α have been known to gradually induce various proinflammatory proteins, such as cytokines IL-6, chemokines IL-8, prostaglandin E2 (PGE2), and interferon which encouraged the progression of OA cartilage [1]. A crucial step of OA treatment is to stop progression of OA by discontinuing the function of inflammatory signal molecules. A therapeutic manner of exogenously administered MSC to OA is supporting anti-inflammatory cytokines to halt action of IL-1 β and TNF- α . As known, AF-MSC can also secrete a variety of secretory cytokine, including IL-10, IL-13, IL-4, and IL-1receptor antagonist (IL-1ra) to counteract inflammatory cytokines [14, 38]. IL-10 is a major endogenous regulator of inflammatory cytokines produced by T cells and macrophage, and thought to be the most effective to reduce IL-1 β and TNF- α . Expression of IL-4 has been found to be a unique characteristic of AF-MSC [14]. The presence of IL-4 was found to reduce the secretion of IL-1 β and TNF- α by activated monocyte at almost 100%, the secretion of IL-6 at 70-85%, as well as the secretion of monokines [39, 40]. IL-4 inhibits the production of proinflammatory cytokines IL-1 β , TNF- α , IL-6, and IL-8 in inflammatory disease [41]. IL-4 and IL-10 can also co-function to inhibit interferon- γ , whereas, IL-4 and IL-13 enhance the synthesis of IL-1ra.

In our study of cartilage tissue outgrowth under either inflammatory cytokines or AF-MSC secretome, we found that cartilage slices incubated under inflammatory medium showed earlier CPC outgrowth than those found in secretome supplemented medium. Within five days of culture, the cartilage tissue cultured under inflammatory medium (four from 48 cartilage slices) and control medium (six from 48 slices) showed the outgrowth, whereas, cartilage outgrowth was not found in tissues cultured under the secretome-supplemented medium. The outgrowth cartilage (one from 48 slices) was found at day 10 in culture of secretome medium. This event might be explained by the homeostasis mechanism in OA cartilage. Homeostasis of cartilage refers to catabolic and anabolic balance. In healthy cartilage, the extracellular matrix (ECM) structure has a low turnover of matrix molecules with a balance of anabolic signal, such as TGF- β and IGF-1, and catabolic signal, such as MMP, IL-1, and iNOS. In the early period of OA, cartilage microenvironment is fulfilled with catabolic signal proteins. Cartilage also elevates anabolic proteins at this period to accommodate homeostatic balance, such as TGF- β and IGF-1. TGF- β , a major anabolic protein induces chondrogenesis via phosphorylation of Smad1, Smad5, and Smad8 in Smad pathway, which influences the promotion of ECM and collagen synthesis, chondrocyte differentiation, and control cartilage degrading enzymes [42]. At this period, quiescent CPC are stimulated for cell proliferation as seen in chondrocyte clusters formation. The cells secrete both matrix-forming proteins

and matrix-degrading proteins [28, 30]. For this reason, it might be possible that cartilage tissues make a homeostatic balance of anabolic expression under inflammatory medium and then are presented in outgrowth cartilage tissues. This idea was supported by the evidence that CPC under secretome supplemented medium showed enhancing of *TGFβ1* and *IGF1*, but also the rise of *MMP13* catabolic gene expression (data not shown). It is also consistent to the description in Lotz *et al.*, who present that chondrocyte cluster expresses both catabolic and anabolic factors [43].

Articular cartilage is a unique feature of a cell and ECM components cross-talking in a specific niche, for example, TGF-β, which is produced by chondrocytes in an inactive form, is secreted, and bind rapidly to ECM to become an active form. Activated TGF-β can be released from ECM and freely diffuse to induce chondrocytes to synthesis ECM components. The specific niche or microenvironment was created by signal molecules from cell-ECM interaction and well-synchronized acts as regulatory factors for restoring and remodeling articular cartilage. Imbalance of signal molecules and homeostasis in cartilage can produce a loss of a specific niche factor and is harmful for cartilage repair. Hence, the target goal of OA cartilage repair might be composed of two steps, a halting inflammation and a recovering homeostasis.

Conclusions

From our study, we conclude that AF-MSC secretome has a great potential as a therapeutic agent for cell-based OA therapy. It contributes a therapeutic response by stop action of IL-1β and TNF-α cytokines in OA inflammation, return property of tissue repair, and play a role in reversing a balance of anabolic and catabolic signal molecules for cartilage homeostasis.

Abbreviations

OA: Osteoarthritis; IL-1β: Interleukin-1β; TNF-α: Tumor necrosis factor α; CPC: Chondrocyte progenitor cells; iNOS: Inducible nitric oxide synthase; COX-2: Cyclooxygenase 2; MSC: Mesenchymal stem cells; AF-MSC: Amniotic fluid-derived MSC; IL-4: Interleukin-4; NF-κB: Nuclear factor-κB; MAPK: Mitogen-activated protein kinase; PDT: Population doubling time; Col I: Collagen type I (gene); RUNX2: Runt-related transcription factor 2 (gene); SOX9: SRY-box transcription factor 9 (gene); Col II: Collagen type II (gene); IGF1: Insulin-like growth factor 1 (gene); TGFβ1: *Transforming growth factor beta* 1 (gene); ERK: Extracellular signal-regulated kinase.

Declarations

Ethical Approval and consent to participate

The consent document was approved by the Ethics Committee of Siriraj Hospital, Mahidol University, Thailand.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SK, KC, NT, BC, and TP contributed to the conception and design of the experiments. SK and PP performed the experiments and acquired the data. SK and TP analyzed and interpreted of the data and drafting of the article. KC, NT, BC, SJ, and TP contributed to technical support and advisement and provision of study materials. All authors have given final approval of the article.

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Figures

Figure 1

Effect of IL-1 β , TNF- α and AF-MSC secretome to CPC migration.

(A) Expanded CPC migrated from cartilage tissue was presented at day five of culture (i), The CPC at subculture passage three presented in a fibroblastic morphologic appearance using inverted microscopy at a 10X magnification (ii). (B) Cell amount in migrating area was observed at day seven of scratch

assay. Experiment was done in triplicate and presented into mean $\pm$ SEM. The significant difference was provided as compared to cell in control medium. CPC in IL-1 β at a concentration of 0.2 and 1 ng/ml or TNF- α at a concentration of 0.5, 5, and 10 ng/ml presented significantly lose migration property. (C) Migratory CPC under control medium (i), inflammatory medium (ii), secretome supplemented medium (iii), moved across the scratching line on day seven of experiment (n = 5). Superiority of AF-MSC secretome medium to motivate CPC migration was found since day three of experiment (iv). The values represent by mean $\pm$ SEM with * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 2

Expression of NF- κ B and MAPK signaling proteins.

CPC was incubated with inflammatory cytokine for 3 h before incubated under CC medium (group 2) or AF-MSC secretome medium (group3) and then cytoplasmic and nuclear extraction for protein analysis by western blot. CPC under CC medium acted as a control (group1). The cytoplasmic and nuclear protein fractions were used to detect NF- κ B (A), pNF- κ B (A), p38 (B), pp38 (B), ERK (C), pERK (C), COX-2 (D), and iNOS (E) protein levels. Relative protein levels in cytoplasmic and nuclear fraction were normalized with actin and histone (F), respectively. ERK, COX-2, iNOS expression in whole cell (cytoplasmic and nuclear) of inflamed CPC was presented (G). Data were presented by mean $\pm$ SEM of three independent experiments. * $P < 0.05$.

Figure 3

Morphology and character of outgrowing CPC.

(A) Outgrowth cartilage and outgrowing CPC under control-, inflammatory-, secretome-medium observed using inverted microscopy with a 10X magnification at day 14 and day 21 of culture. Cell senescence and apoptosis presented in CPC under inflammatory medium, whereas, CPC under control medium and secretome medium had high proliferation and migratory potential. (B) Forty-eight cartilage slices in each medium were observed for a presenting of CPC outgrowing. The outgrowth tissues were counted and compared among three studied media. (C) Amount of CPC derived from cartilage slices cultured under different medium. The cell amount presented in mean $\pm$ SEM, ** $P < 0.01$.

Figure 4

Effect of IL-1 β , TNF- α and AF-MSC secretome to molecular change in CPC.

Expression levels of chondrogenic genes; *Col I*, *Col II*, *RunX2*, *Sox9* (A) and cartilage anabolic genes; *IGF-1*, *TGF- β* (B) were analyzed in CPC cultured under control-, inflammatory- and secretome-medium for 96 h

using quantitative real-time RT-PCR. Experiment was done in triplicate and data were presented by mean $\pm$ SEM. Significant value was not found by statistical analysis among cells in different medium.

Figure 5

Action of AF-MSC secretome to ERK signaling pathway.

With appearance of IL-1 β /TNF- α combination, production of ERK protein in CPC cytoplasm was promoted and induces phosphorylation on ERK protein to presenting high level of pERK. AF-MSC secretome acts to inhibit the production of ERK and the phosphorylation and translocation of pERK to nucleus leading to inhibit COX-2 and iNOS production.