Repair of Partial Thickness Cartilage Defects Using Cartilage Extracellular Matrix Membrane-Based Chondrocyte Delivery System in Human *Ex Vivo* Model

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Treatment options for partial thickness cartilage defects are limited. The purpose of this study was to evaluate the efficacy of the chondrocyte-seeded cartilage extracellular matrix membrane in repairing partial thickness cartilage defects. First, the potential of the membrane as an effective cell carrier was investigated. Secondly, we have applied the chondrocyte-seeded membrane in an *ex vivo*, partial thickness defect model to analyze its repair potential. After culture of chondrocytes on the membrane *in vitro*, cell viability assay, cell seeding yield calculation and cell transfer assay were done. Cell carrying ability of the membrane was also tested by seeding different densities of cells. Partial defects were created on human cartilage tissue explants. Cell-seeded membranes were applied using a modified autologous chondrocyte implantation technique on the defects and implanted subcutaneously in nude mice for 2 and 4 weeks. *In vitro* data showed cell viability and seeding yield comparable to standard culture dishes. Time dependent cell transfer from the membrane was observed. Membranes supported various densities of cells. *Ex vivo* data showed hyaline-like cartilage tissue repair, integrated on the defect by 4 weeks. Overall, chondrocyte-seeded cartilage extracellular membranes may be an effective and feasible treatment strategy for the repair of partial thickness cartilage defects.

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Key Words: Partial thickness cartilage defect; Cartilage extracellular matrix membrane; Autologous chondrocyte implantation; Cartilage repair

INTRODUCTION

Partial thickness cartilage defects (PTCD) are common predisposing lesions to osteoarthritis [1,2]. These lesions are observed in the form of cracks, fissures and fibrillations during arthroscopy of the knee joint, corresponding to International Cartilage Repair Society (ICRS) grade 1 or 2 lesions [1]. The most perplexing feature of these defects is that they rarely heal spontaneously and are prone to propagate into larger, full thickness cartilage defects (FTCD) [1,3]. Unlike FTCDs, the marrow space of PTCDs is not breached. Bone marrow residing cells requisite for repair, therefore, cannot reach the defect. Articular cartilage itself is a hostile environment for repair, due to its hypo-

cellular, avascular, and anti-adhesive properties. Remaining cartilage on the surface of PTCDs contain anti-adhesive dermatan sulfate and other proteoglycans which hinder adhesion of recruited cells requisite for repair [4]. These unique features of PTCDs call for novel treatment approaches [5,6].

Several proof-of-concept studies have reported early results for the repair of PTCDs using strategies to overcome the anti-adhesive property of remnant cartilage. These include the use of chondroitin sulfate-based bioadhesives or matrix degrading chondroitinase ABC [1,7,8]. Such strategies, however, have yet to show consistent hyaline-like cartilage repair.

Clinically verified strategies for cartilage repair such as microfracture, autologous chondrocyte implantation (ACI), and mosaicplasty are currently applicable on FTCDs only. ACI, nevertheless, may be a promising strategy for PTCDs. It has resulted in some improvement in the quality of repair in an equine PTCD model [9]. In another study, successful cartilage repair was achieved by modification of ACI using chondrocyte sheets

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cultivated in temperature-responsive culture dishes [10].

A number of research groups have developed collagen based biomembranes for cell therapy of cartilage defects. Most are designed for the use in ACI, in order to replace the periosteum as cell coverage material [11,12]. Ideally, they hold and protect implanted chondrocytes from synovial fluid, while providing a scaffold for cellular attachment and growth. We have recently developed a cartilage extracellular matrix (CECM) membrane, which contains natural articular cartilage components. This material has shown good tensile strength, cytokine permeability, biocompatibility, and hyaline cartilage-like repair potential in rabbit and canine microfracture models [12,13].

The purpose of this study was to evaluate the efficacy of the chondrocyte-seeded CECM membrane in repairing PTCDs. First, the potential of the CECM membrane as an effective cell carrier was investigated. Secondly, we have applied the chondrocyte-seeded CECM membrane in an *ex vivo*, PTCD model to analyze its repair potential.

MATERIALS AND METHODS

Preparation of cartilage extracellular matrix membrane

All experimental protocols involving the use of animals were approved by the Animal Care and Use Committee of our institution. CECM membranes were prepared as described previously [10]. Briefly, articular cartilage was obtained from stifles of 3 week old pigs (Farmsco Co., Seongnam, Korea). Cartilage pieces were treated with 0.2% collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA) for 18 hours. After digestion, cell pellets were washed twice with DMEM and seeded in 6-well-tissue culture plates (TCP) at a density of 7.5×10^5 cells/well. After 2 weeks, cultured chondrocytes and extracellular matrix (ECM) were collected. Decellularization of the cell-ECM complex was done by sequential treatment with 0.3% sodium dodecyl sulfate solution 3 times for 2 hours at RT, followed by 200 U DNase solution for 2 hours at 37°C. Decellularized ECM was washed with and spread out to form membranes. The thickness of the CECM membrane ranged from 30 to 60 μm .

Isolation and culture of chondrocytes

Knee joints were harvested from 4 different 2-week-old male New Zealand white rabbits (Orient Bio, Seongnam, Korea). Articular cartilage from the femoral condyles were dissected and minced followed by digestion with 0.2% collagenase type II. Chondrocytes were seeded in DMEM supplemented with 10% (v/v) fetal bovine serum, and 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA) at a cell density of 7×10^3 cells/cm². Chondrocytes were used after two passages. Cells were seeded

on CECM membranes at a cell density of 2×10^5 cell/cm² and were cultured for 3 days. As controls, chondrocytes were cultured in standard TCP under the same condition.

Cell viability assay

Viability of chondrocytes cultured on TCP and CECM membrane was compared by the Live/Dead Viability/Cytotoxicity Kit (Invitrogen, Carlsbad, CA, USA).

Cell seeding yield

Cell seeding yield on TCP and CECM membrane was compared. After 3 days of culture, medium was removed in each group. Each dish was rinsed 3 times with 5 mL of PBS. Rinsed PBS was collected from each dish and centrifuged. Unattached chondrocytes were counted 3 times each by 2 different observers. The number of unattached cells was subtracted from the initially seeded cell number.

Surface morphology of chondrocyte-seeded CECM membrane

The morphology of chondrocytes seeded on the CECM membrane was observed using scanning electron microscope (SEM) after 3 days of culture.

Cell transfer assay

Cell transfer assay was done to assess chondrocyte transfer potential of the CECM membrane. Chondrocytes were seeded on CECM membranes at a cell density of 2×10^5 cell/cm². After 3 days of culture, chondrocyte-seeded membranes were turned upside down onto TCPs so that cells were in contact with the culture dish surface. Afterwards, cultures were further carried out for 2, 3, and 5 days. Migration and proliferation away from the CECM membrane was visualized by confocal microscopy.

High density culture

To evaluate the potential of the CECM membrane's cell carrying ability, chondrocytes were cultured in different cell densities on the CECM membrane. Cells were labeled with red fluorescent PKH26 (Sigma-Aldrich, St. Louis, MO, USA). CECM membranes were placed on TCPs coated with fibrin glue (Greenplast®, Green Cross, Seoul, Korea) on the peripheral rim, so that the center of the membrane became concave. Cells were seeded on CECM membranes at cell densities of 2×10^5 cell/cm², 4×10^5 cell/cm², and 6×10^5 cell/cm². Each group was cultured for 6 days. Samples were fixed in 4% paraformaldehyde for 24 hours and cryo-sectioned for fluorescence microscopy.

Ex vivo cartilage tissue repair system

All experimental protocols using human tissue samples were

approved by our Institutional Ethical Board Review. PTCDs were made in osteochondral explants (1×1×1 cm) from human lateral femoral condyle (LFC) cartilage obtained from patients who had undergone total knee arthroplasty. Explants were extracted from areas where cartilage was grossly intact. A custom made surgical tool (Hankiltech, Hwaseong, Korea) that makes multiple cuts 1 mm deep into the cartilage was used to create PTCDs (Fig. 1A). Three explants were harvested from each patient's LFC and were allocated into three groups; defect alone, CECM membrane, and chondrocyte-seeded CECM membrane. In the 'defect alone' group, PTCDs were created without any additional procedures. In the 'CECM membrane' group, PTCDs were covered with membranes, and edges were sealed with fibrin glue. In the 'chondrocyte-seeded CECM membrane' group, chondrocyte-seeded membranes were placed on the PTCDs, with the cell-seeded surface facing the defect (Fig. 1B and C). Edges were subsequently sealed with fibrin glue. Tissue explants from all three groups were surgically inserted in the back of nude mice and cultivated for 2 and 4 weeks. A total of 42 different LFC samples from 14 different patients, 7 patients for each time point, were used in this study.

Histological analysis and cell tracking

After tissue retrieval at respective time points, specimens were fixed in 10% formaldehyde for 5 days. Specimens were processed and embedded in paraffin. Five, 10 µm thick sections, each 20 µm apart, were cut from the partial thickness defects. Slides were stained with Safranin-O/Fast Green (SAFO). Histological scoring of the defect site was done using the ICRS assessment scale for cartilage repair by 2 blinded observers who did not participate in this study [14]. In order to track migration of membrane-seeded chondrocytes after *ex vivo* implantation, chondrocytes were labeled with PKH26. After labeling, the same protocol of the *ex vivo*, 'chondrocyte-seeded CECM membrane' group was followed. This PKH 26-labeled *ex vivo* group was also observed at both 2 and 4 weeks post surgical implantation. Each specimen was cryosectioned after harvest. Background was stained with DAPI and slides were observed with

confocal microscopy.

Statistical analysis

Each *in vitro* experiment was done at least 4 times. All data are presented as mean±95% confidence intervals (95% CI). Mean values of each group were compared with Mann-Whitney U-test for statistical significance for the *in vitro* experiments. Data of *ex vivo* experiments were compared by Kruskal-Wallis test with Dunn's multiple comparison test.

RESULTS

CECM membrane is highly compatible with chondrocytes for culture

Quantification of viability revealed that viable cells on TCP and CECM membrane accounted for a mean of 97.07±2.31% and 97.04±2.87%, respectively ($p=0.5626$) (Fig. 2A). As for cell seeding yield, 86.67±5.41% and 85.37±6.34% of chondrocytes were attached on TCP and CECM membrane, respectively ($p=0.8857$) (Fig. 2B). SEM images showed even distribution of cells and newly synthesized ECM on the membrane (Fig. 3).

Figure 2. Biocompatibility of cartilage extracellular matrix (CECM) membranes. (A) Quantification of viable cells after cell seeding in tissue culture plates (TCP) and CECM membranes ($p=0.562$). (B) Cell seeding yield after seeding in TCPs and CECM membranes ($p=0.885$).

Figure 1. Methodology of *ex-vivo* experiment. (A) Gross image of partial defects (arrow) made on the human cartilage explants. (B) Gross image after cell-seeded membrane application (arrow) on the partial defects. (C) Schematic representation of the 'cartilage extracellular matrix (CECM) membrane' group (left) and 'chondrocyte-seeded CECM membrane' group (right). Arrow indicates the CECM membrane.

CECM membrane supports transfer of chondrocytes towards surrounding environment

Cell transfer assay revealed a time dependent migration and proliferation of chondrocytes from the CECM membrane to-

wards TCP *in vitro*. Attachment of cells on TCP around the periphery of the CECM membrane was observed as early as day two. By day 5, transfer distance of chondrocytes tripled compared to day 2 (Fig. 4).

Figure 3. Scanning electron microscopic images of cartilage extracellular matrix (CECM) membrane surfaces with and without cells. (A) Surface morphology of CECM membrane without cells. (B) CECM membrane after 3 days of cell culture. Even distribution of cells and attachment of chondrocytes to the surface of the membrane were observed. Scale bars=50 μm.

Figure 4. Cell transfer assay. Chondrocytes exhibited time dependent migration and proliferation away from the cartilage extracellular matrix (CECM) membrane towards tissue culture plate (TCP). Upper left corner area in each figure indicates the cell-seeded CECM membrane turned over towards the TCP. White arrow indicates the transfer distance from the CECM membrane.

CECM membrane is suitable for carrying various dosages of cells

At 2×10^5 cell/cm², chondrocytes attached to the membrane in a single layer. At 4×10^5 cell/cm², chondrocytes aggregated in

the center of the membrane, forming a mound of cells. The base of the mound was uniformly attached to the membrane. At 6×10^5 cell/cm², the mound of cells increased in size, compared to other densities (Fig. 5).

Figure 5. Chondrocyte seeding at different densities on the cartilage extracellular matrix (CECM) membrane. At 2×10^5 cells/cm², chondrocytes seeded in a single layer on the CECM membrane. At densities greater than 4×10^5 cells/cm², chondrocytes stacked on top of each other forming a mound of cells. Top row shows bright field microscopic images and bottom row shows confocal microscopic images of PKH26-labeled chondrocytes. Magnification, $\times 40$.

Figure 6. Representative histological results of ex vivo experiment. Each row shows images of the cartilage defects stained in Safranin-O/Fast Green. Hyaline-like cartilage repair with integration on the defect can be observed in the chondrocyte-seeded cartilage extracellular matrix membrane group at 4 weeks. Magnification, $\times 40$, $\times 100$, and $\times 400$.

The cell-seeded CECM membrane repairs PTCs in an *ex vivo* model

Two weeks after the *ex vivo* experiment, the 'defect alone'

Figure 7. International Cartilage Repair Society (ICRS) scoring of *ex vivo* histological results. *p* values >0.05 among each group are shown in the graph. Error bars indicate 95% confidence intervals. CECM: cartilage extracellular matrix.

group showed no signs of cartilage repair on SAFO slides. Fibrillations, fissures and decreased matrix staining with sparsely distributed chondrocytes were noted. After 4 weeks, these degenerative changes progressed, with further decrease in matrix staining. As for 'CECM membrane' group, there were also no signs of cartilage repair. At 2 weeks, the surface was relatively smooth and a stronger matrix staining was noted compared to the 'defect alone' group. At 4 weeks, however, matrix staining decreased with no signs of repair. The CECM membrane itself also decreased in thickness and surface became irregular, a sign of material degradation. No signs of inflammatory reactions were noted around the membrane. As for 'chondrocyte-seeded CECM membrane' group, signs of early cartilage repair were evident by week 2. Chondrocytes filled the pits formed in the partial thickness defect. The matrix, however, was still irregular, with absence of definitive glycosaminoglycan deposition. By 4 weeks, hyaline cartilage-like repair was achieved. Numerous chondrons were evenly distributed throughout the matrix. Intense staining of the matrix was noted, suggestive of hyaline cartilage repair. Well integrated margins were also noted (Fig. 6). ICRS scoring revealed significantly higher scores for 'chondrocyte-seeded CECM membrane' group compared to the other two groups at both 2 and 4 weeks ($p=0.0065$, $p=0.0012$). ICRS scores at 2 weeks for defect alone, CECM membrane, and chondrocyte-seeded CECM membrane were 0.833 ± 1.03 , 1.33 ± 1.62 ,

Figure 8. Cell tracking after *ex vivo* transplantation of the cell-seeded, cartilage extracellular matrix (CECM) membrane group. PKH26-labeled chondrocytes were found near the surface of the defect, away from the CECM membrane post 2 and 4 weeks transplantation. Background staining done with DAPI. Magnification, $\times 200$. DAPI: 4', 6-diamidino-2-phenylindole.

and 5 ± 1.97 , respectively. At 4 weeks, respective scores were 1.166 ± 0.84 , 2.66 ± 1.32 , 10 ± 1.04 (Fig. 7).

The implanted cells of the 'chondrocyte-seeded CECM membrane' group were tracked with PKH26. Labeled cells were identified at both 2 and 4 weeks. Implanted cells were found throughout the defect, including the surface of the host cartilage. Implanted cells, however, did not penetrate host cartilage tissue (Fig. 8).

DISCUSSION

This study sought to evaluate the potential of the CECM membrane as a cell carrier and its effectiveness in repairing PTCDs. *In vitro* data shows that this membrane is an effective cell carrier, evident by its biocompatibility and support of cell transfer towards the surrounding environment. *Ex vivo* data shows that chondrocyte-seeded CECM membrane successfully adhered to the damaged surface of PTCDs, resulting in hyaline cartilage-like repair. Our findings provide a proof of concept in treating PTCDs using cell-seeded CECM membrane transplantation.

A major obstacle in developing PTCD repair technology is the anti-adhesiveness of the remaining cartilage, which hinders the integration of implanted cells or tissue. Proteoglycans such as biglycan and decorin provide the cartilage with anti-adhesive properties [1,4,15]. In order to overcome this obstacle, various strategies have been studied. Material wise, Wang et al. [8] have achieved integration of poly(ethylene glycol)diacrylate hydrogel into cartilage through photopolymerization. Another strategy is to apply enzymes on the cartilage surface. Jürgensen et al. [16] have shown that transglutaminase could be used as a 'biological glue' in PTCDs. Chondroitin ABC has been used to remove glycosaminoglycan chains of proteoglycans, in turn, reducing anti-adhesiveness of remaining cartilage [1,6,15]. While increased recruitment and attachment of synovium-derived stem cells in the defects have been reported, the most recent study failed to show stem cell differentiation into hyaline cartilage and integration in the PTCD model [7]. A recent article reported that the approach using enzymatic degradation of cartilage matrix in fact did not enhance the integration of newly formed cartilage [17]. We were able to achieve integration of transplanted chondrocytes into the PTCDs without the help of enzymes or subsequent manipulation of the implanted material. All anchorage dependent cells such as chondrocytes possess intrinsic adhesive properties when cultivated *in vitro* [18]. By cultivating chondrocytes on the CECM membrane and subsequently turning over the material towards the defect, intrinsic adhesive properties of chondrocytes were most likely preserved. A similar concept has been report-

ed by Kaneshiro et al., [10] where chondrocyte sheets were engineered from temperature-responsive culture dishes. These dishes allowed detachment of chondrocytes without disrupting the ECM. Chondrocyte sheets integrated well into the PTCDs, resulting in cartilage repair.

The use of CECM membranes in PTCDs may also provide protection against inflammation. Migration of macrophages together with synthesis and deposition of matrix-metalloproteinase-9 have been observed in a porcine PTCD model [19]. Such inflammatory infiltrations into the cartilage matrix and newly synthesized matrix further degrade cartilage and impair integration of repaired cartilage into the host tissue. A previous study showed that the CECM membrane provided protective coverage for the defects in a bone marrow stimulation rabbit model [13]. Simply covering the PTCDs with the membranes resulted in small improvement in ICRS scores (1.166 ± 0.84 and 2.66 ± 1.32 , $p=0.076$) at 4 weeks. These results suggest that the CECM membrane may not only provide protection against leakage of implanted cells, but also protection against macrophages and subsequent inflammation.

Based on published evidence, the most feasible strategy for treating PTCDs so far may be the ACI technique. ACI has been clinically validated by numerous groups for treating FTCDs [20-22]. Nixon et al. [9] have applied this technique in an equine PTCD model and observed improvement in histological repair scores. Sophisticated tissue engineering strategies have been applied to overcome limitations of the original ACI technique. Many biomembranes have been developed to replace the periosteum as coverage material [23]. The MACI® technique (Genzyme, Norden, The Netherlands), ChondroGide® (Geistlich, Wolhusen, Switzerland), and Maix® (Matricel, Hezugenrath, Germany) all utilize a bilayer collagen I/III matrix biomembrane for the treatment of FTCDs. These biomembranes, however, have not been validated for the treatment of PTCDs. Our results provide a proof-of-concept that these newer generations of ACI may be effective in the treatment of PTCDs.

The strengths of our study are as follows. First of all, the CECM membrane was highly compatible with chondrocytes. Maintenance of normal chondrocyte phenotype, although not shown in this study, has been demonstrated previously in a 3D scaffold using the same material [23]. Furthermore, the CECM membrane is manufactured by decellurization of cartilage ECM. Although no comparative studies have been done, this membrane is expected to provide a more native environment for chondrocytes compared to previous collagen I/III based biomembranes. Secondly, the CECM membrane functioned well as a cell carrier. *In vitro* results demonstrate that the membrane supports various cell densities, allowing cultivation and transfer of chondrocytes. Most importantly, we have combined

a feasible, validated surgical procedure (ACI) with chondrocyte-seeded CECM membrane to treat PTCDs. Material properties of the CECM membrane allows for easy handling, barrier function from synovial fluid, and cytokine permeability [12]. Integration of neo-cartilage tissue into the PTCDs and hyaline-like cartilage repair has been achieved, without the use of degrading enzymes acting on the surrounding matrix.

There are several limitations in this study. First of all, materials, cells, and tissue originating from different species were used in the *ex vivo* experiment. Although signs of rejection such as graft failure or inflammatory cell infiltration were not observed, we cannot rule out the effects of xenogenic transplantation. Previous studies, however, have not reported rejection related changes during implantation of xenogenic tissue engineered constructs within an ECM surrounding, such as PTCDs [24,25]. The CECM membrane, furthermore, has recently been cleared for human use by the Ministry of Food and Drug Safety in our country. Secondly, although ACI-treated cartilage explants have been validated under *ex vivo* conditions in nude mice, this *ex vivo* model does not entirely replicate the *in vivo* joint environment [26]. Our previous results with the CECM membrane, however, suggests that the membrane can withstand the *in vivo* joint environment in rabbit and canine models, resulting in favorable results after microfracture [12,13]. Further studies incorporating quantitative, as well as qualitative analysis of the newly formed cartilage matrix in an *in vivo* animal model are needed.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

This study was approved by the Institutional Review Board of Ajou University School of Medicine.

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