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Inhibition of blood vessel formation by a chondrocyte-derived extracellular matrix

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ABSTRACT

In this study, the chondrocyte-derived extracellular matrix (CECM) was evaluated for its activity to inhibit vessel invasion *in vitro* and *in vivo*. Human umbilical vein endothelial cells (HUVECs) and rabbit chondrocytes were plated on a bio-membrane made of CECM or human amniotic membrane (HAM). The adhesion, proliferation, and tube formation activity of HUVECs and chondrocytes were examined. The CECM and HAM powders were then mixed individually in Matrigel and injected subcutaneously into nude mice to examine vessel invasion *in vivo* after 1 week. Finally, a rabbit model of corneal neovascularization (NV) was induced by 3-point sutures in the upper cornea, and CECM and HAM membranes were implanted onto the corneal surface at day 5 after suture injury. The rabbits were sacrificed at 7 days after transplantation and the histopathological analysis was performed. The adhesion and proliferation of HUVECs were more efficient on the HAM than on the CECM membrane. However, chondrocytes on each membrane showed an opposite result being more efficient on the CECM membrane. The vessel invasion *in vivo* also occurred more deeply and intensively in Matrigel containing HAM than in the one containing CECM. In the rabbit NV model, CECM efficiently inhibited the neovessels formation and histological remodeling in the injured cornea. In summary, our findings suggest that CECM, an integral cartilage ECM composite, shows an inhibitory effect on vessel invasion both *in vitro* and *in vivo*, and could be a useful tool in a variety of biological and therapeutic applications including the prevention of neovascularization after cornea injury.

1. Introduction

Articular cartilage is an avascular tissue and vessel invasion is a big challenge for the repair of damaged cartilage. Vessel invasion can severely affect normal function of the cartilage by reducing the matrix synthesis, causing apoptosis of cells and eventually accelerating cartilage degeneration in osteoarthritis [1,2]. To overcome this limitation, the articular cartilage is known to have intrinsic angiogenesis inhibitors such as chondromodulin-I, endostatin and thrombospondin-1 [3]. Chondromodulin-I regulates the vessel invasion during endochondral bone formation and inhibits endothelial cells growth [4,5]. Endostatin inhibits the migration of endothelial cells in response to vascular endothelial growth factor (VEGF) and angiogenesis [6,7]. Thrombospondin-1 induces apoptosis of endothelial cells via CD36 binding and subsequent activation of $p59^{fyn}$ and caspse-3-like protease but is also known to inhibit endothelial tube-like structure formation via binding with decorin, a cartilage-enriched proteoglycan [8–10]. In addition to these extracellular matrix (ECM) fragments, the type II collagen-

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derived N-terminal propeptide (PIIBNP) was also shown to have anti-angiogenic activity *in vitro* [11]. However, it is present only in the type IIA procollagen that is mainly expressed in chondroprogenitors and in fetal cartilage tissue [12]. Chondroitin sulfate (CS) in the articular cartilage was also shown to inhibit endothelial cell adhesion and induce anti-angiogenic and anti-inflammatory responses in chondrocytes *in vitro* [13,14]. Many previous studies have demonstrated that ECM components play significant roles in regulating growth and morphology of vessel formation [15,16]. During angiogenesis, endothelial cells are stimulated by angiogenic factors, and undergo a cascade of events including increased proliferation, degradation of ECM of surrounding tissues for endothelial cell migration, recruitment, interconnection and lumenization [17,18]. In all these processes, the interaction of ECM and endothelial cells not only modify the angiogenic behavior of endothelial cells but also influence their response to anti-angiogenic factor [19,20]. Considering these results and those of the above studies on the cartilage ECM *in vitro*, it is highly plausible that the cartilage ECM can directly inhibit angiogenesis.

We have previously fabricated a sponge-type scaffold made solely of chondrocyte-derived ECM (CECM) [21]. Porcine chondrocytes were cultured in a 3-dimensional (3-D) environment without an ectopic scaffold and the resulting construct was subjected to freeze-drying to remove cells and produce micropores in it. The CECM scaffold consisted of newly synthesized cartilage ECM and was shown to have much better activity than polyglycolic acid (PGA) scaffold in cartilage tissue formation using chondrocytes and mesenchymal stem cells (MSCs) [21–23]. In particular, the engineered cartilages using MSCs in the CECM scaffold showed much less hypertrophic degeneration and vessel invasion *in vivo* than those in the PGA scaffold [23–25]. This phenomenon might attribute to the anti-angiogenic activity of chondrocytes differentiated from MSCs, which was much better in the CECM scaffold than in the PGA scaffold [23]. However, it is also possible that CECM itself inhibited directly the vessel invasion into the construct, which needs further studies to elucidate. In this study, we hypothesized that the CECM alone could inhibit activity of endothelial cells and vessel formation *in vitro* and *in vivo*. The CECM scaffold was prepared in thin film and powder forms, and compared with that of human amniotic membrane (HAM) on the adhesion and proliferation of endothelial cells and chondrocytes *in vitro*. In addition, we evaluated that the effect of CECM on the vessel invasion into the gel-type scaffold in nude mice and on the suture-induced corneal neovascularization (NV) in rabbits.

2. Materials and Methods

2.1. Cell culture

HUVECs purchased from Lonza Walkersville, Inc (Walkersville, MD, USA) were cultured in Endothelial Cell Basal Medium-2 (EGM-2) (Clonetics, Walkersville, MD, USA) at 37 °C under 5% CO_2. Cells from passages 4–7 were used. For all experiments, HUVECs were grown to 70%–80% confluence and made quiescent by starvation for at least 18 h. Rabbit chondrocytes were freshly isolated from rabbit articular cartilage, obtained from knee of 2-week-old female ($n = 1$) New Zealand white rabbits. Cartilage pieces were digested in 1 mg/mL collagenase (Worthington, Somerville, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA). Isolated cells were passed through a nylon mesh cell strainer (BD Biosciences, Bedford, MA, USA) and centrifuged at 1700× *g* for 10 min. Cell pellet was resuspended in DMEM with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 1% antibiotic-antimycotic. The culture medium was changed every three day.

2.2. Preparation of CECM and HAM in film and powder forms

A thin film shape of the CECM scaffold was prepared as described previously [26]. In brief, primary chondrocytes from porcine knee joints were cultured in monolayer at high density of 3×10^6 cells/mL for 3 weeks to form a cell-ECM composite membrane. The membrane was then treated with DNase I and sodium dodecyl sulfate (SDS) to remove cell components, and finally fabricated to a thin film of 30–60 μm in thickness. The HAM film was purchased from Bioland (Cheonan, Korea). The films was cut by biopsy punch to make their diameter same as 8 mm. To produce powder forms, CECM and HAM films were frozen at −70 °C, freeze-dried, and comminuted to a particulate form using a cryogenic sample crusher (JFC-300, JAI, Japan). All products were sterilized by EO gas for 24 h at 27 °C before use.

2.3. Adhesion and proliferation assay for chondrocytes and HUVECs

For cell adhesion assay, rabbit chondrocytes or HUVESs were seeded on the CECM and HAM films in 24 well plates at 1×10^3 cells per well. After 24 h, non-adherent cells were removed and adherent cells were counted to calculate the number of attached cells. In addition, cells were also pre-stained with calcein AM (2 μg/mL; Invitrogen, Carlsbad, CA, USA) and the amount of cells attached was measured by their fluorescent intensity at 494 nm using a microplate reader (Infinite M200, Tecan, CH). For cell proliferation assay, 1×10^3 cells were seeded on the CD-ECM and HAM films in 24 well plates. After 1 h, non-adherent cells were removed and adherent cells were further cultured for 7 days with the culture medium replenished at days 3 and 5. The amount of cells proliferated was measured at 1, 4 and 7 days by WST assay (EZ-cytox; Daeil Lab Service, Seoul, Korea). The adhesion and proliferation assays on conventional cell culture plates without films were used as controls.

2.4. Tube formation assay

The CECM and HAM powders were mixed with Geltrex (Invitrogen) at 15.4 mg/mL and the mixture were coated on the 6 well plates. HUVECs were seeded on the plates at a density of 1.9×10^4 cells per well and cultured in the medium supplemented with 100 ng/mL bFGF. After 24 h, the formation of tube-like structures was observed under the bright field and fluorescence microscopes after calcein AM staining. For quantitative analysis, images were captured from 10 random microscopic fields per well, and the number and length of tubes were measured using Image J program.

2.5. Vessel invasion assay in athymic nude mice

Male athymic nude mice at six weeks of age were obtained from Taconic Farms, Inc. (Germantown, NY, USA). The experimental protocol followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Animal Care and use committee of Ajou University. Matrigels (BD Biosciences) containing CECM or HAM powder (3 mg/250 μL) was injected into the back of nude mice subcutaneously. Matrigels without any ECM materials were injected as a control. Three samples from each group were implanted in a mouse longitudinally along the side of the spine. Total 15 constructs were implanted in 5 mice ($n = 5$). Mice were sacrificed at 1 week for gross observation and histological analysis of the implanted constructs.

2.6. Analysis of implanted samples

After implantation in the nude mice for 1 week, the samples were retrieved and examined for the appearance and vessel infiltration via gross observation and histological analysis. The samples were fixed in 4% formaldehyde for 24 h, dehydrated, and then embedded in paraffin wax. Sections of 4 μm in thickness were prepared and first stained with heamtoxylin & eosin (H&E). For immunostaining, the sections were treated sequentially with 3% H_2O_2 for 10 min, 0.15% Triton X-100 for 10 min, and 1% BSA in PBS for 10 min. Then, they were incubated with primary antibodies (1:200) for 1 h at room temperature. The primary antibodies used were against alpha smooth muscle actin (α-SMA; ab5694), CD 31 (PECAM; l ab28364), HIF-1α (ab1), VEGF (ab1316) and endostatin (ab53702) (all from Abcam, Cambridge, UK). After washing 3 times in PBS, the sections were incubated with a biotinylated secondary antibody against mouse IgG (1:200) for 1 h and peroxidase-conjugated streptavidin solution for 30 min at room temperature (SPlink HRP Detection Kit; Golden Bridge International, City of Industry, CA, USA). The sections were finally counterstained with Mayer's hematoxylin (Sigma, St. Louis, MO, USA) and then mounted for microscopic observation (Nikon E600, Japan).

2.7. A Rabbit model of corneal neovascularization (NV)

This study was conducted in accordance with the Guidelines for Animal Experiments approved by Inje University College of Medicine (No.; 2012-028) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Nine male New Zealand white rabbits, weighting between 2.0 kg and 2.5 kg were obtained from Samtako (Osan, Korea). Systemic anesthesia was induced by intramuscular injection of the mixture of Zoletil (Virbac Laboratories, Carros, France), at a 0.2 mg/kg dose, and topical anesthesia was induced by proparacaine eye drop of Alcaine (Alcon Inc., Seoul, Korea). A corneal suture of 3 mm in length was performed through the corneal stroma area at 1 mm distance from the corneal limbus using 7-0 mersilk (Ethicon, Somerville, NJ, USA). After 5 days, formation of the corneal neovascularization (NV) was confirmed and the suture was removed. The rabbits were randomly divided into three groups of the untreated control group and the CECM or HAM implanted group ($n = 3$/group). The CECM and HAM films were implanted onto the corneal surface to completely cover the resected area and fixed with 10-0 nylon sutures (Ethicon). Normal rabbits were also used as a vehicle group ($n = 3$). The rabbits were sacrificed at 10 days after implantation. Clinical evaluation