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Original Article

Behavior of Fibroblasts on a Porous Hyaluronic Acid Incorporated Collagen Matrix

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A hyaluronic acid (HA) incorporated porous collagen matrix was fabricated at -70℃ by lyophilization. The HA incorporated collagen matrix showed increased pore size in comparison with collagen matrix. Biodegradability and mechanical properties of matrices were controllable by varying the ultraviolet (UV) irradiation time for cross-linking collagen molecules. Addition of HA to collagen matrix did not effect ultimate tensile stress after UV irradiation. HA incorporated collagen matrices demonstrated a higher resistance against the collagenase degradation than collagen matrix. In an *in vitro* investigation of cellular behavior using dermal fibroblasts on the porous matrix, HA incorporated collagen matrix induced increased dermal fibroblast migration and proliferation in comparison with collagen matrix. These results suggest that the HA incorporated collagen porous matrix assumes to enhance dermal fibroblast adaptation and regenerative potential.

Key Words: Hyaluronic acid, atelocollagen, biodegradation, mechanical properties, ultraviolet irradiation, fibroblast growing behavior

INTRODUCTION

Reconstruction of an extracellular matrix (ECM) is an important process in healing of deep skin defects involving dermis.[1] Biomaterials used as wound dressing for tissue regeneration are usually required to biodegrade in the tissue and simultaneously regenerate tissue.[2] Currently available materials include collagen,[3-5] gelatin,[6] hyaluronic acid,[7,8] chitosan,[9] polyglycolic acid, polylactic acid and their copolymers.[10,11] Among these materials, collagen, a major component of ECM, is considered to be the most promising biomaterial for skin regeneration due to many desirable properties, e.g., controllable biodegradation, low antigenicity, minimal inflammatory and cytotoxic properties and the property of promising cellular growth.[12-15] Hyaluronic acid (HA), an unbranched polysaccaride consisting of repeated β-D-glucuronic acid/β-N-acetyl-D-glucosamine disaccharide units, is ubiquitously present in the ECM of many tissues such as skin and cartillage. HA is considered as a space-filling, structure-stabilizing, cell-coating, and cell-protective polysaccharide. It forms a structurally integrated system with the fibrous proteins in the intercellular space, creating the elastoviscous, protective, lubricating, and stabilizing matrix in which cells are embedded. Its exceptionally high rheological property, a highly hydrated random coil with very large molecular volume, provides the hydration of ECM and permits the metabolites to interpenetrate at relatively low concentration by diffusion.[16] It is also known to stimulate the proliferation and migration of a large variety of cell types and to improve the wound healing because of its interaction with cells.[17] Current strategies for tissue regeneration focus on the extension of cell-matrix basic scientific concepts for development of implantable matrices to mimic natural tissues.

Three-dimensional construction of collagen based dressing has been reported to canalize

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fibroblast migration from wound through its porous structure, leading to produce ECM newly synthesized by cells.[18] Fabrication of porous structure like sponge has been achieved in several ways, in particular, by freezing and lyophilization.[18,19] However, collagen based sponge has some limitations because it is particularly susceptible to enzymatic digestion and has weak mechanical stability.[20] Introduction of crosslinking is effective in reduction of the biodegradation rate, preventing rapid leaching of the material into wound fluids.[20] Collagen molecules are usually crosslinked by covalent bonds after glutaraldehyde (GA), formaldehyde, diisocyanate or acyl azide treatments.[21-24] Particularly, GA treatment has been an effective crosslinking method of collagen based biomaterials specifically for implantation. However, this treatment results in cytotoxicity due to residual aldehydes and heterogeneous crosslinking which cause only crosslinking of the material surface, especially in heart valves.[25] In order to avoid the additional washing steps and to ensure the absence of toxic materials, physical crosslinking methods such as ultraviolet (UV) irradiation, dehydrothermal (DHT) treatment, have been used as alternative methods.[26-29]

Both collagen and glycosaminoglycans such as chondroitin-6-sulfate and HA have extensive histories in a wide variety of biomedical applications.[17-22] HA and HA derivatives are available in a wide range of physical forms (fluids, gels and solids) and have been used as a biomaterial for joint surgery, tissue engineering and drug delivery.[16,30] However, there has been a little report on the fabrication and physico-chemical characterization of HA impregnated collagen material for skin regeneration.[31] This study was purposed to investigate the possibility of using a HA incorporated collagen porous matrix for tissue regenerative scaffold. Two types of porous matrices were fabricated and crosslinked by UV irradiation: collagen and HA incorporated collagen. These matrices were characterized with respect to the pore morphology, resistance against collagenase activity, mechanical properties, cell adhesion, proliferation, and migration.

MATERIALS AND METHODS

Fabrication of HA incorporated collagen matrix

The type I atelocollagen was extracted from bovine tail skin using pepsin and salt precipitation as previously described.[32] The collagen precipitate was lyophilized at -40℃ and kept at 4℃, before use. The type I atelocollagen was dissolved in 0.001 N HCl solution at 4℃ and adjusted to pH 7.4 by adding 1N NaOH solution. The solution was centrifuged at 3,000g, 4℃ for 15 min, and 5 ml of the resulting precipitate was poured onto Petri-dish (5 cm in diameter) to fabricate porous collagen matrix by freezing at -70℃ and lyophilization at -50℃. In order to fabricate HA incorporated collagen porous matrix, HA (sodium salt, MW = 1,200-1,500 kD, Hanwha Groups, Taejon, Korea) was mixed to the concentrated collagen precipitate with the weight ratio of HA to collagen to be 2:8, and then was poured onto a Petri-dish and freezed at -70℃ and lyophilyzed at -50℃.

Crosslinking of collagen matrix

The matrices were crosslinked by ultraviolet (UV) irradiation (wave length 254 nm). The collagen matrix was placed in a transparent polymethylmethacrylate (PMMA) cases, and then delivered to a self-designed UV chamber (Daeil DBO 231S, Seoul, Korea). The specimens were exposed to surrounding eight 10 W UV bulbs for 1/2, 2, 4 h at 4℃ under N_2 purging. The distance between the light source and sample was 5 inches, and intensity of the UV was 1.66 mW/cm^2 equivalent to 93.3 mJ/cm^2/min.

The morphology of produced matrix was observed by a scanning electron microscope (SEM) (JSM 54300, JEOL Ltd, Tokyo, Japan). The average diameter of pores in matrix was measured by using a computerized image analyzer (Bum-Mi Universe Co. Ltd, Ansan, Korea).

Mechanical testing of collagen membranes

Mechanical properties of the matrix crosslinked by UV irradiation were determined to investigate the extent of crosslinking. The porous matrix was prepared as strips (10 × 4 × thickness mm), and

uniaxial tensile strength was tested by a material testing machine (Micro Bionix, Tryton, MN, USA). Specimen's ends were fixed using Teflon grips and abrasive paper, as described previously.[33] The thickness of individual matrix was measured using a micrometer (Mitutoyo Co., Kanagawa, Japan). The load cell capacity for detecting forces was 10 N. Strain was expressed as the change in length as a percentage of the initial length. All tests were carried out at room temperature. The data were stored from the load cell and linear variable differential transformer by the data acquisition software of Teststar 4.0 (MTS Systems Co, Tryton, MN, USA). The sampling rate was 5Hz. From the load-displacement relationship obtained by above system, stress-strain curve was extracted. Ultimate tensile stress (UTS) was determined from the stress-strain relation.

In vitro degradation of crosslinked porous matrix

The degradation of crosslinked porous matrix was investigated by using bacterial collagenase (Type IA , Sigma, St. Louis, MO, USA). Five mg of matrix was incubated for 5 h at 37℃ in 2 ml of collagenase solution [10 units/ml in 0.05 mM Tris-HCl buffer, pH 7.4 (Sigma, St. Louis, MO, USA)] containing 10 mM $CaCl_2$. The extent of crosslinking was determined by measurement of collagen amount dissolved in solution using Bradford method.[34] In order to examine the morphological change of fabricated membranes, matrix (1 cm in diameter) was stained by 50 μl of 0.2% crystal violet in 2% ethanol solution, and was freeze-dried. The stained matrix was photographed after degradation test.

Isolation of fetal dermal fibroblasts

Primary cultures of fetal dermal fibroblasts were obtained by sequential enzymatic treatment of fetus, following the method described by Linge et al.[35] Fetal skin was aseptically dissected from a fetus obtained on May 1998 from a therapeutic abortion performed on a 35-year-old Korean female at the Department of Obstetrics and Gynecology, Severance Hospital, Seoul, Korea. After permission from the Medical Resource Committee at the Yonsei University College of Medicine, the epidermal layer was striped off. Tissue was minced and digested at 37℃ in the enzyme mixture of 0.1% collagenase and 0.25% trypsin in 0.02% EDTA (Sigma, St. Louis, MO, USA) with magnetic stirring. After digestions, the cells were collected by centrifugation for 10 min at 400 × g. The cell pellets were resuspended into 35 mm tissue culture dishes at a density of 4×10^4 cells/cm^2 in Dulbecco's modified Eagle's medium containing 10% FBS and 1% antibiotic-antimycotic solution (FBS-DMEM) (Sigma, St. Louis, MO, USA). Cultures were maintained in a humidified atmosphere consisting of 95% air and 5% CO_2 at 37℃. Confluent monolayers were propagated by trypsinization (0.25% trypsin, 0.02% EDTA) and replating at 1 : 2 dilution. For experiments, fibroblasts were used in passages 5 - 9.

Cell adhesion and proliferation

Specimens to study cell adhesion were divided into three groups; tissue culture plates (TCPs) as the control, porous collagen matrix, and HA incorporated collagen porous matrix. Each specimen was prepared to oval shape with diameter of 1.2 cm. Specimens were thoroughly washed by PBS solution and placed onto tissue culture plates (24 wells). Each 1.2×10^5 fibroblasts were seeded on a specimen, and cultured for 3 h. By washing with phosphate-buffered saline (PBS) solution, unattached cells were removed. After incubation in 0.5 ml of enzyme mixture (0.1% collagenase and 0.25% trypsin in 0.02% EDTA) for 20 min, the adherent cells were removed from the substrate and then the specimens were thoroughly washed twice by 0.5 ml of PBS solution. Cells in enzyme solution and PBS solution were centrifuged together for 10 min at 400 g, and then resuspended in a fresh PBS solution. An aliquot of the resulting cell suspension was counted by a hematocytometer.

To investigate proliferation of fibroblast on the collagen and HA incorporated collagen porous matrices, specimens were placed onto the bottom of TCPs (48 wells), and fixed by an inert, silicone-based, vacuum grease to prevent them from floating in the growth media. Fibroblasts were plated on the matrix with a plating density of 2.5×10^4, and the medium was changed every 24 h.

Cell proliferation on each specimen was determined after 1, 3, 5, and 7 d. The specimens were gently washed with PBS to remove any unattached cells. The attached cells were removed from the matrix by incubation in 200 μl of mixed enzyme solution for 20 min and then discs were washed with 200 μl of PBS solution. Cells in enzyme solution and PBS solution were centrifuged together for 10 min at 400 g, and then re-suspended in fresh PBS solution. An aliquot of the resulting cell suspension was counted using hemacytometer. All the measured values were statistically analyzed by a paired students's t-test (Excel 5.0, Microsoft, WA, USA).

Cell migration

In vitro cell migration was investigated by a self-designed hemi-cylinder, which was made of PMMA (16 mm in diameter) (Fig. 1). A porous collagen matrix was placed in a well (24 wells) and then PMMA hemi-cylinder was placed on the top of matrix. Fifty thousands cells were seeded on the top of matrix which was not covered by hemi-cylinder. The matrix was pre-incubated for 2 d at 37℃ to allow confluent cell monolayer on the substitute. After incubation, PMMA hemi-cylinder was removed and then the matrix was further incubated for 2 d at 37℃ to allow cell migration. The migrated cells from boundary of cell monolayer were stained by Toluidine Blue, and were observed by a light microscope.

RESULTS AND DISCUSSION

Fabrication of HA incorporated collagen porous matrix

The collagen and HA incorporated collagen matrices were fabricated by freezing at -70℃ and lyophilization at -50℃. The produced matrix was three-dimensionally porous with 111 ± 32 μm and 180 ± 28 μm pore sizes, respectively, and an interconnected network of collagen matrix was observed (Fig. 2). The freezing process initially produced the nuclei of ice crystallization, and the nuclei gradually increased to final size of ice crystals, and the frozen materials are dried with sublimating ice crystals under vacuum at a temperature below the ice freezing temperature.[36] It is reported that a rapid cooling rate causes formation of many nuclei of ice crystals, resulting in formation of smaller-sized pores.[37,38] Therefore, the differences in the pore size between the collagen and HA incorporated collagen matrices demonstrates the differences in heat transfer rates between two matrices during the freezing process of primary collagen solutions.[37] It has been reported that the early extraction of the crystallizing

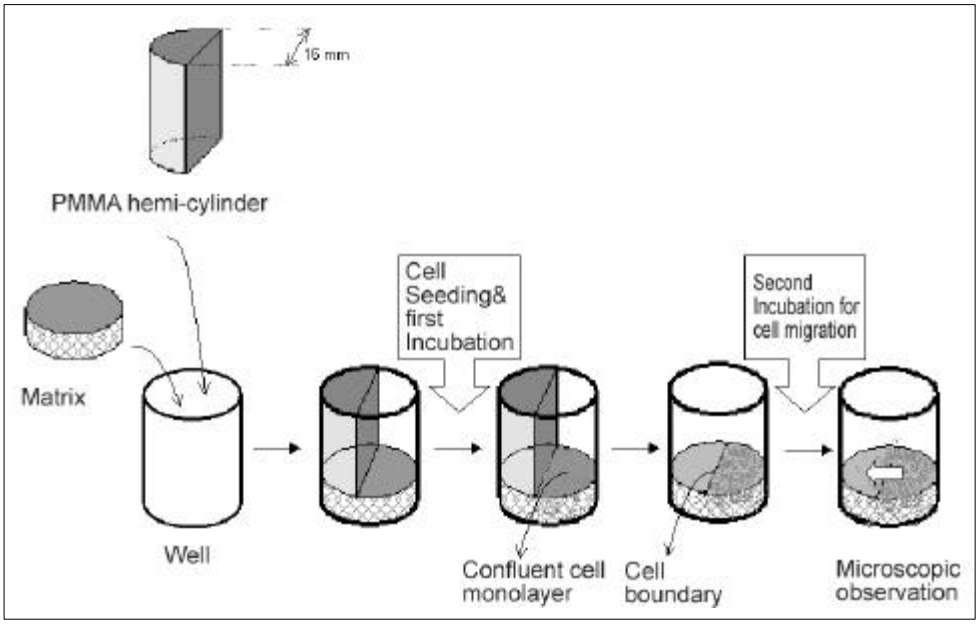

Fig. 1. Experiment process of migration test.

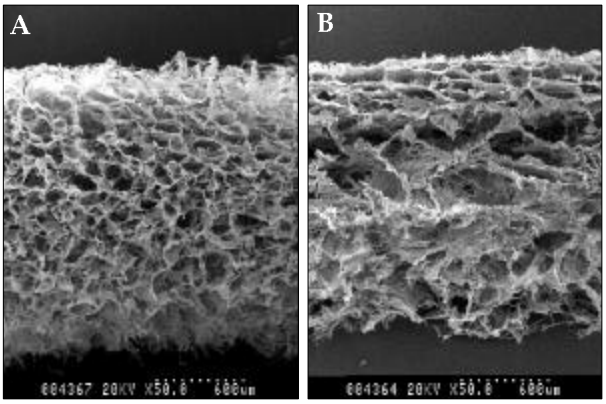

Fig. 2. Structures of (A) collagen and (B) HA incorporated collagen porous matrix observed by SEM.

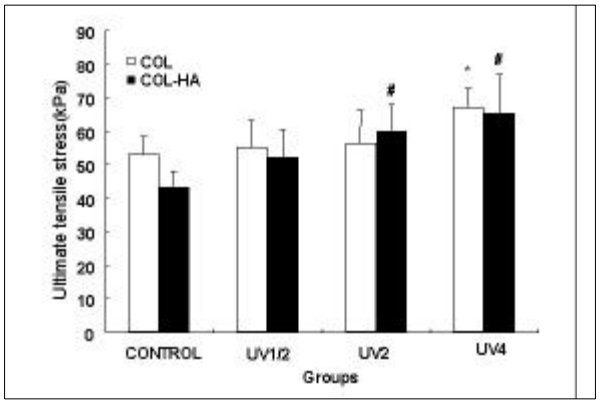

Fig. 3. Ultimate tensile stress of collagen (COL) and HA incorporated collagen matrix (COL-HA) in relation to UV irradiation time; Each point represents the mean ± SD kPa in 5 specimens. Control: non-treated group, UV1/2: UV irradiated group for 1/2 h, UV2: UV irradiated group for 2 h, UV4: UV irradiated group for 4 h.

heat can prevent the large ice crystal formation by controlling freezing temperature, addition of organic solvents, and property of the primary solution.[18,19,37,38] The difference in pore size between two matrices assumes to be related to that addition of HA into the primary collagen solution would reduce freezing rate of the solution due to highly viscoelastic and unusual rheological property of HA. Ionic interaction between HA and collagen might also produce the larger porosity in matrix, because the crosslinking occurred directly between HA and the collagen molecules without any additional organic compound had led to coprecipitation with consequently producing phase separation.[31] Besides the biological effect of adding HA in respect to dermal cell infiltration and proliferation into porous collagen matrix, addition of HA into the primary collagen solution would play an important role in regulating the pore size and structure. This result was supported by the reports that incorporation of HA into gelatin matrix could increase porosity of matrix.[31,39]

Mechanical properties of the matrix

Ultimate tensile stress (UTS) of the matrix after crosslinking was investigated (Fig. 3). The UV treatment for 4 h increased UTS of both collagen and HA incorporated collagen matrices in comparison with control groups, and this result is supported by the reports that collagen molecule could be crosslinked by UV irradiation.[26,27,40,41] Although addition of HA reduced the initial UTS, UV irradiation regained UTS. It is possible that HA provides highly hydration to collagen matrix and resulted weak mechanical property. However, UTS of HA incorporated collagen matrix increased significantly after UV treatment for 2 h as well as for 4 h, differently from collagen matrix. This result suggests that combination of HA and collagen provides favorable environment to the matrix for photochemical crosslinking. UV (wave length: 254 nm) irradiation produces radicals in the form of unpaired electrons in nuclei of aromatic residues such as those in tyrosine and phenylalanine, and binding of these radicals result in collagen crosslinking.[20] Therefore, it is important to provide an environment necessary for photochemical reaction among the produced radicals. Menter et al. reported that the radicals are short-lived to be quenched by molecular O_2.[42] Thus, we performed UV irradiation under N_2 gas purging to remove the radical scavenger O_2. Our results showed that HA also contributed to prevent the radicals from rapid disappearance owing to its high viscosity that made HA to physically protect radical's movement.

As an ideal wound dressing for skin regeneration, matrix should have efficient flexibility, durability, adherence, and ability to absorb wound debris and to prevent wound's dehydration. In order to possess these properties, the matrix should also have proper mechanical properties that maintain their shape during *in vivo* appli-

cation. As well as tensile stress, strain % of matrix was observed. In UV4 h groups, except of HA incorporated matrix, there was no significant difference among the specimens (Fig. 4).

In vitro biodegradability

To predict biodegradability of the porous collagen matrix, the dissolved amount of collagen from matrix in collagenase solution was investigated. Generally, collagenase is known to cleave amide bonds within the triple helical structure of type I collagen, and has specificity for the proline (Pro)-Y-glycine (Gly)-proline (Pro)-Y' region, splitting Y and Gly.[43] The dissolved collagen amounts from both the UV irradiated collagen and HA incorporated collagen specimens were less than that of the control group (Fig. 5). This result indicates that the higher degree of crosslinking produced the higher resistance to collagenase digestion. HA incorporated collagen matrix showed less dissolved collagen amount than collagen matrix with indicating that HA incorporated collagen porous matrix was highly crosslinked by UV irradiation. Since collagen molecule has only 51 aromatic residues out of the 3156 amino acid residues and consequently crosslinking degree by UV irradiation is limited in a matrix, ionic interaction between HA and collagen led to higher resistance against collagenase activity.[20,31] These results could be supported by microscopic observation (Fig. 6).

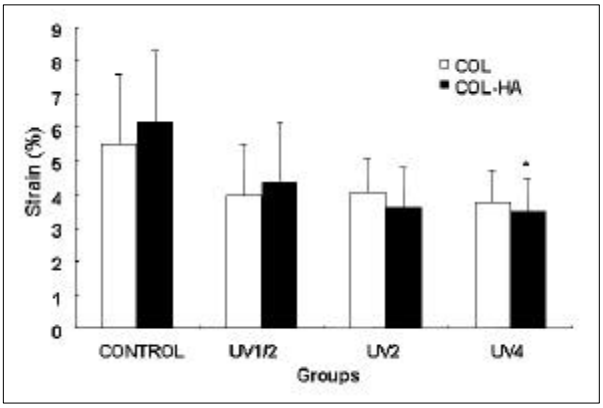

Fig. 4. Strains of collagen (COL) and HA incorporated collagen matrix (COL-HA) in relation to UV irradiation time; Each point represents the mean ± SD % in 5 specimens. Control: non-treated group, UV1/2: UV irradiated group for 1/2 h, UV2: UV irradiated group for 2 h, UV4: UV irradiated group for 4 h.

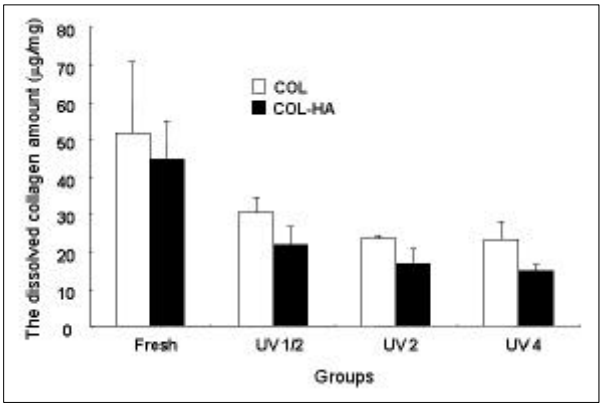

Fig. 5. Enzymatic degradation of matrix in relation to UV irradiation time.; Each point represents the mean ± SDμg per mg in 5 specimens. Control: non-treated group, UV1/2: UV irradiated group for 1/2 h, UV2: UV irradiated group for 2 h, UV4: UV irradiated group for 4 h.

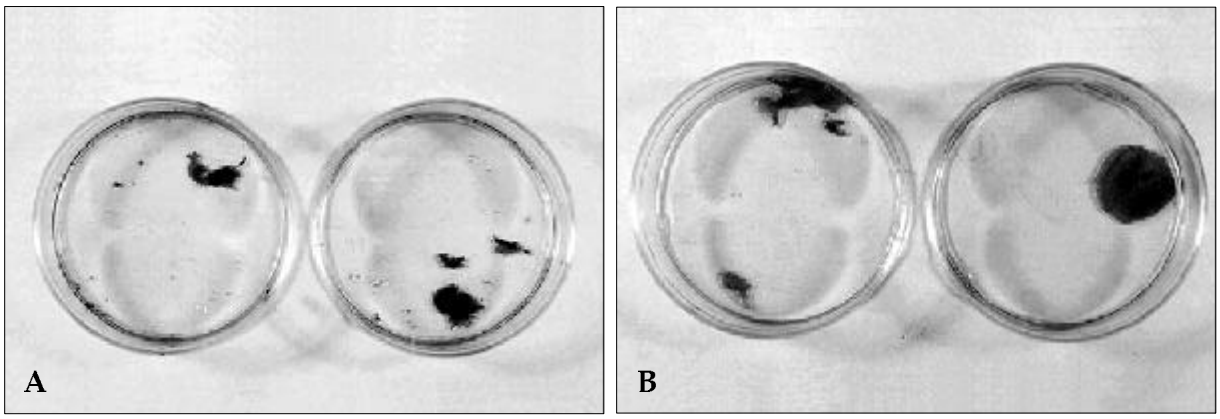

Fig. 6. Light micrograph of matrix biodegradaded in collagenase solution. (A) collagen matrix (left: non-treated group, right: UV irradiated group) and (B) HA incorporated collagen matrix (left: non-treated group, right: UV irradiated group).

Cell adhesion and proliferation

Cell attachment onto the collagen and HA incorporated collagen porous matrix was investigated to understand the interaction of their surfaces with cell (Fig. 7). The number of attached cells on collagen and HA incorporated collagen matrix was significantly higher than the control group in which cells were cultured on the conventional culture plate ($p < 0.05$). Collagen is an extracellular matrix that has cell adhesive properties, and collagen coating on polymer scaffold surface for tissue engineering resulted in rapid cellular adhesion.[44] The initial attachment reaction of most cultured cell is based on interaction between arginine-glycine-aspartic acid ligand on collagen molecules and $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrin on cell surface. The two integrins mediate transmembrane transduction of signals that ultimately leads to changes in gene expression patterns of fibroblasts.[45] The mechanism of cell attachment in this case may involve the formation of focal contacts through a clustering of cell integrin receptor on the cell surface that interact with collagen.[43,44] From our results, collagen matrix would play a useful role to support the initial attachment and growth of cells.

Proliferation of dermal fibroblasts cultured on the specimen was observed for 1, 3, and 7 d (Fig. 8). The number of proliferated cells in both collagen and HA incorporated collagen matrices was gradually increased as the culture time courses, significantly after 4 h. This result demonstrates that two matrices have proper porous capacity for proliferation and infiltration of cells. Since skin is a vascular tissue, porosity has to provide sufficient space for neovascularization in the newly generating tissue in the substitute.[18] In general, it has been considered that fibroblast proliferation and function was enhanced in three-dimensional cultures with spongy matrices of pore diameter above 100 μm.[18,37]

Addition of HA to collagen matrix produced the higher number of cells on 1, 3, 5, and 7 d after incubation period in comparison with collagen matrix. However, no significant difference was observed in the cell growth rate on between two groups ($p > 0.05$), and it does not also conclude that HA enhanced cell proliferation rate up to 7 d, because there was no difference between two matrices. Considering the number of cells attached to collagen matrix for 1 d was significantly lower than HA incorporated matrix ($p < 0.05$), it could be anticipated that fibroblasts can freely migrate into HA incorporated collagen matrix. This hypothesis was investigated through the following migration test.

Cell migration

The HA incorporated collagen porous matrix induced more fibroblasts infiltration into matrix than the collagen matrix (Fig. 9). This result suggests that HA accelerated the motion of cell into

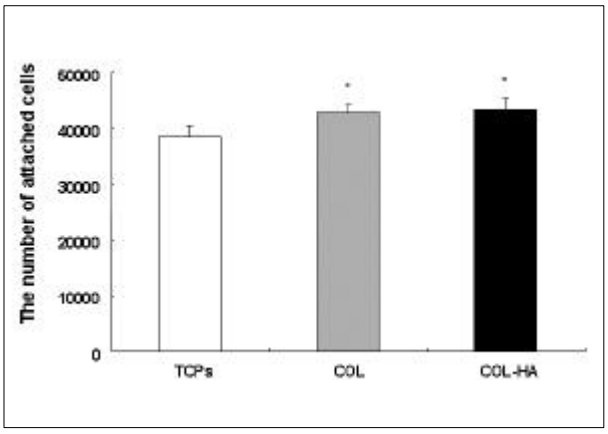

Fig. 7. Adhesion of fibroblasts onto the collagen (COL) and HA incorporated collagen matrix (COL-HA): TCPs was used as control. Each point represents the mean ± SD cells in 4 samples.

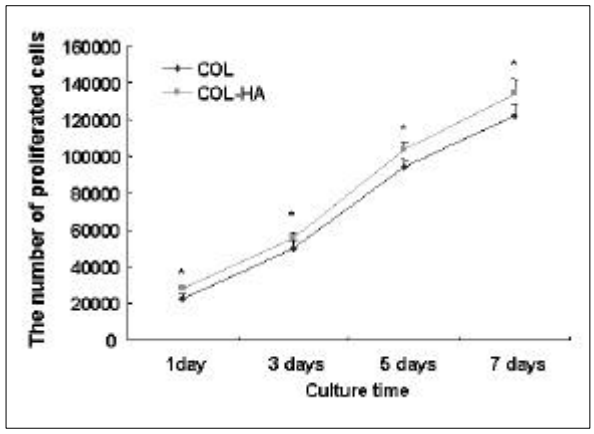

Fig. 8. Proliferation kinetics of fibroblasts cultured in the collagen (COL) and HA incorporated collagen matrix (COL-HA). Each point represents the mean ± SD cells in 4 samples.

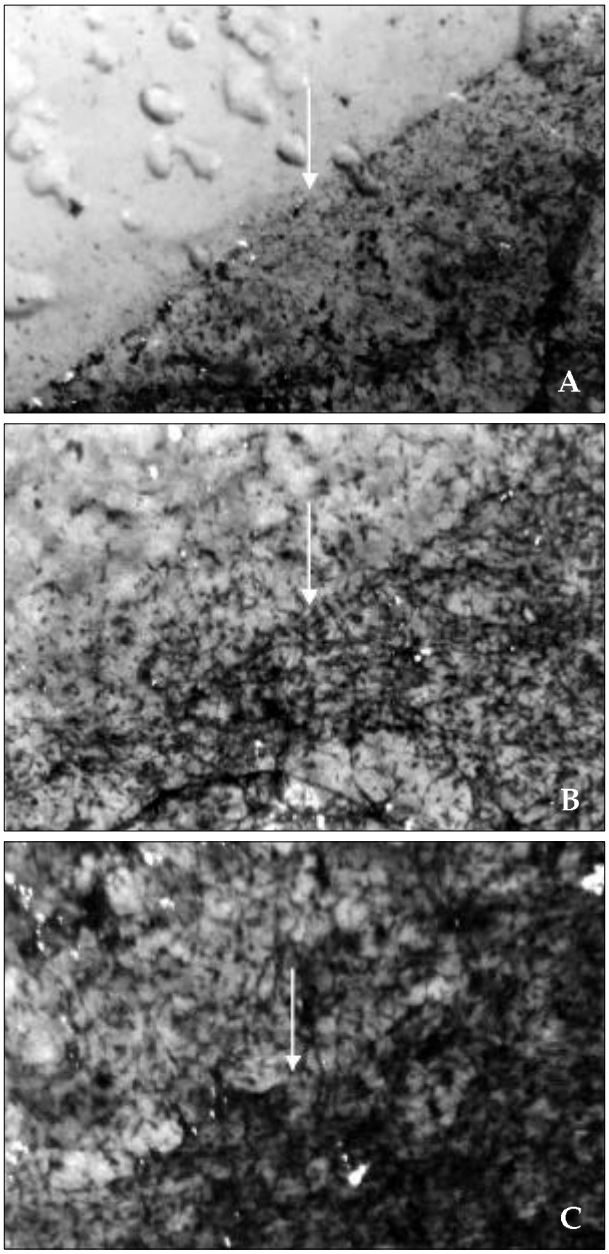

Fig. 9. Migration of fibroblast on the matrix by microscopic observation (A) collagen matrix before migration test, (B) the collagen and (C) HA incorporated collagen matrix after migration test. Arrows indicate cell boundaries.

porous structure owing to its high elastoviscosity, lubricating property. And the unique rheological property from very large molecular volume helps cell motility and permits unhindered diffusion of metabolites between the cells.[16] In addition, interactions between HA and specific receptors on the cell surface have effects on the various cellular functions involving cell proliferation and activation, cell-cell interaction, and migration.[17] The HA-binding molecule on cell surface is CD44, a broadly distributed cell surface glycoprotein with multiple isoforms generated by alternate splicing of at least 10 exons encoding the membrane-proximal portion of extracellular domain. The amino terminus of CD44 displays 30% homology with link protein tandem repeats and contains the HA-binding domain. Another representative cell surface HA is a fibroblast protein known as RHAMM, an acronym for the receptor for HA-mediated motility.[30] Based on these findings, HA would cause rapid cell migration within the matrix, leading to reconstruction of connective tissue. These results demonstrated that HA incorporated collagen porous matrix can be utilized as material with cellular growth-increasing potential, and would be a useful tool in improving tissue-regenerating efficacy as a scaffold for tissue engineering.

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