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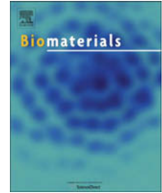

The growth of a vascular network inside a collagen–citric acid derivative hydrogel in rats

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ABSTRACT

Three-dimensional regenerative tissue with a certain bulk cannot survive without sufficient blood perfusion in vivo, so construction of a vascular system in regenerative tissue is a key technology in tissue engineering. In order to construct such a vascular system, we attempted to create a scaffold material that induces neovascular growth from the recipient bed into the material. This material, an ion complex gel matrix (IC gel) consisting of collagen and a citric acid derivative, enabled it to associate with basic fibroblast growth factor (bFGF). The IC gel was implanted in the subfascial space of the rat rectus muscle and excised 5 days later. Cross-sections of the excised samples were stained for von Willebrand factor, and then neovascular development into the gel was observed and also quantified by image analysis. These data showed that the IC gel markedly induced growth of vascular-rich tissue into the inside of the gel by day 5, which surpassed that after implantation of Matrigel® or gelated collagen. Further, combination with bFGF significantly enhanced the vascularization ability of IC gel. These findings suggest that IC gel functioned as a scaffold material for neovascular ingrowth and a reservoir of bFGF.
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1. Introduction

Construction of a vascular system in regenerative tissue is a key technology in the field of tissue engineering, since highly-organized regenerative tissue cannot survive in vivo without sufficient blood perfusion. Cultured cells and small amounts of tissue can attain an adequate nutrient supply and waste transfer through the mechanism of passive diffusion. However, when three-dimensional (3-D) tissue with a certain bulk is implanted under physiological conditions in vivo, passive diffusion from the recipient bed is generally limited to the surface of the tissue structure, indicating that blood perfusion via a vascular system is required to maintain the viability of the whole tissue [1–4].

To construct a vascular system in regenerative tissue, the most practical approach could be induction of neovascular growth from the surrounding host vasculature into the regenerative tissue. In this case, construction of a vascular network should be completed in the orders of days, since bulky regenerative tissue without a vascular system cannot maintain viability in vivo for a longer period than that. A well-established method for induction of vascular formation is delivery of an angiogenic growth factor, such as vascular endothelial growth factor or basic fibroblast growth factor (bFGF) [5]. Previous studies demonstrated that local delivery of such factors successfully promoted neovascularization in native tissues and organs, and this method has been applied to the treatment of several types of ischemic disease [6–8]. However, in regenerative tissue, there is a more critical requirement in realizing neovascular formation, which is a scaffold structure for vessels. Vessels in vivo are essentially within the extracellular matrix (ECM), which functions as a natural scaffold for the vessel structure. Vessels must be supported by ECM to maintain their structure, and vascular cells receive various signals and stimulation via ECM [9–12]. To date, few regenerative tissues that contain an ECM-like component for vascular formation have been developed [10,13]. The scaffold structure for vessels must then be suitably furnished to achieve construction of a vascular system in the regenerative tissue.

The purpose of this study was to create a scaffold material that rapidly induces neovascular growth from the recipient bed into the inside of the material. In our previous study, we reported that a biopolymer, alkali-treated collagen modified with a citric acid derivative (AlCol-CAD), had high affinity to vascular endothelial cells in vitro, with no toxicity [14–16]. AlCol-CAD potentially forms

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a hydrogel matrix by forming an ion complex with atelocollagen (AtCol). This ion complex gel (IC gel) possesses a stable 3-D matrix structure and enables a reserve of bFGF, a potent angiogenic growth factor.

2. Materials and methods

2.1. Preparation of ion complex gel matrix

The preparation of AlCol-CAD and AtCol was reported previously [15,16]. In brief, AlCol and AtCol derived from pig tissues were provided by Nitta Gelatin Inc. (Osaka, Japan). AlCol, whose isoelectric point is 5, has carboxyl groups generated by the hydrolysis of residual amide groups that exist in asparagine and glutamine of AtCol. Dicyclohexylcarbodiimide (DCC) was purchased from Kokusan Chemical Co., Ltd. (Tokyo, Japan), and other chemicals were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Citric acid was first dissolved in tetrahydrofuran (THF), and then *N*-hydroxysuccinimide and DCC were added. The resulting mixture was stirred for 30 min, and then concentrated by rotary evaporation under reduced pressure to remove THF. The final mixture was recrystallized to yield pure citric acid derivative (CAD). AlCol was first dissolved in dimethyl sulfoxide (DMSO), and then CAD solution was added to synthesized AlCol-CAD matrix. This mixture was subsequently immersed in excess pure water for 48 h at 37 °C to remove DMSO from the AlCol-CAD matrix. AlCol-CAD was finally dissolved in 0.1 M phosphate-buffered solution, pH 8.0, at a concentration of 1% (w/v). AtCol was dissolved in 0.05 N HCl at concentrations of 1% and 2% (w/v). Mixing AlCol-CAD and AtCol at the same volumes, an ion complex hydrogel matrix formed spontaneously at room temperature, since AlCol-CAD is an anion and AtCol is a cation. We prepared two types of IC gel that were found to form stable gel; one was IC[1%] consisting of 1% AlCol-CAD and 1% AtCol, and the other was IC[2%] consisting of 1% AlCol-CAD and 2% AtCol.

2.2. Culture of HUVEC on gel

In order to examine the behavior of vascular endothelial cells on IC gel, human umbilical vein endothelial cells (HUVEC) were cultured on IC[1%] and IC[2%]. Then, their two-dimensional morphology on the gel and number of HUVEC were evaluated. IC gel was poured flat into a 24-well cell culture plate at 170 μL per well (~50 μm thick). HUVEC (2×10^4 cells, Sanko Junyaku Co., Ltd., Ibaraki, Japan) were subsequently seeded on the gel layer and cultured in EGM-2 medium (Takara Bio Inc., Shiga, Japan). HUVEC were washed with 500 μL PBS after 24 h incubation. Then, the two-dimensional morphology of HUVEC on the gel was observed under a phase-contrast microscope by focusing on the surface of the gel. The number of HUVEC on the gel layer was assessed with a Cell Counting Kit-8 (CCK; Dojindo Co., Ltd., Kumamoto, Japan). CCK is a colorimetric assay of cellular dehydrogenase activity, where absorbance at 450 nm is proportional to the amount of dehydrogenase activity in cells [17,18] (each $n = 3$). As controls, the same experiments were carried on layers of normal density Matrigel® (Basement Membrane Matrix whose ingredients and concentration were not modified, BD Biosciences, Bedford, MA) or 1% simple collagen (gelated form, COL[1%]), which was type I collagen solution from pig skin (Nippon Meat Packers. Inc., Tsukuba, Japan). Matrigel® or COL[1%] was poured flat into 24-well culture plates at 170 μL per well (~50 μm thick), and then HUVEC were seeded and cultured in the same manner.

2.3. Implantation of IC gel into subfascial space of rat

All procedures were approved by The Institutional Animal Care and Use Committee. The in vivo ability of neovascular growth into IC gel was evaluated by implanting IC[1%] or IC[2%] into the subfascial space of Wistar inbred rats (male, 300 g, Nippon Bio-Supp. Center Co., Ltd., Tokyo, Japan).

To avoid spread of the implanted gel, a cuff-shaped container (inner diameter 3 mm, width 1 mm), which was the cross-section of a polytetrafluoroethylene vascular conduit (Gore-Tex Stretch Vascular Graft, W.L. Gore & Associates, Inc., Flagstaff, AZ), was filled with IC gel (14 μL) and then placed between the fascia and the muscle of the rectus abdominis. At 5 days after implantation, the rats were sacrificed by intraperitoneal injection of an overdose of 5% pentobarbital sodium 5 days after implantation, and perfusion fixation was then performed by intracardiac injection of 20 mL of 4% paraformaldehyde at 120 mmHg after pre-infusion with lactated Ringer's solution. Since spontaneous vascular growth is generally limited to several tenths of micrometers per day [19], 5 days was considered to be the minimum period for inducing sufficient vascular growth into the whole of the 1 mm-thick material. The implant was then excised with the surrounding tissue, and divided into two pieces in the container to be embedded in paraffin. Other set of rats underwent implantation of a container filled with Matrigel®, COL[1%], or an empty container. Five rats were used in all groups of the experiment.

Further, we implanted IC[1%] into another animal in the same manner, and prepared a sample without perfusion fixation to confirm blood cells in vessels.

2.4. Histological examination

Then, 5-μm sections were cut from each paraffin block and first stained with hematoxylin and eosin (HE). Immunohistochemical staining for von Willebrand factor (vWF) was performed to identify vessels in the implant. After pretreatment with antigen retrieval solution (Dako North America, Inc., Carpinteria, CA), peroxidase blocking reagent (Dako North America), and normal donkey serum solution (1:50, Sigma–Aldrich Inc., St. Louis, MO), rabbit polyclonal antibody against vWF (1:200, Dako North America) was applied for 60 min at 37 °C. Subsequent incubation with biotinylated donkey anti-rabbit IgG (1:100, Chemicon International, Inc., Temecula, CA) and reagents in the ABC Elite Kit (Vector Laboratories, Inc., Burlingame, CA) was performed.

Further, immunostaining for alpha smooth muscle actin (αSMA) was carried out to detect vessels with smooth muscle cells. The sections were incubated with mouse monoclonal antibody against αSMA (1:100, Dako North America) after pretreatment with 0.1% trypsin solution (Roche Diagnostics K.K., Tokyo, Japan) and peroxidase blocking reagent. Then, biotinylated horse anti-mouse IgG (1:200, Vector Laboratories) was applied, and analysis was performed with an ABC Elite Kit.

2.5. Quantitative evaluation of immunostained sections

Photomicrographs of sections stained for vWF were taken with a digital microscope (BZ-900, Keyence, Osaka, Japan) to analyze neovascular development in the implanted material. Since the developed neovasculature in the implant might be heterogeneous in size, wall thickness, tone, etc., three kinds of parameters were calculated. The area for evaluation was set inside the container. The digital data of photomicrographs were first analyzed by ImageJ (National Institutes of Health, Bethesda, MD) [20,21] to measure the number and lumen area of stained vessels, and the vascular wall area of stained vessels was measured using angiogenesis image software (Kurabo, Okayama, Japan) [22–24]. Parameters were calculated as follows:

Vessel number ($/\mu m^2$) = vessel number/analysis area
Vessel area ($\mu m^2/\mu m^2$) = lumen area/analysis area
Vascular wall area ($\mu m^2/\mu m^2$) = wall area/analysis area

Each section stained for αSMA was photographed in the same manner, and the αSMA-positive area was measured using ImageJ, and calculated as follows:

αSMA-positive area ($\mu m^2/\mu m^2$) = αSMA-positive area/analysis area

2.6. Incorporation of bFGF into IC gel

Before gel formation, bFGF (Recombinant Human FGF basic; R&D Systems, Inc., Minneapolis, MN) was added to a mixture of AlCol-CAD (1% and 2%) and AtCol solutions at final concentrations of 10, 100, and 1000 ng/mL. Selection of the bFGF concentration was based on the bFGF concentrations that are generally used to supplement culture media for endothelial cells [25]. Basic FGF-incorporated IC gels were prepared as follows:

1% and 2% IC gels with 10 ng/mL FGF ($IC[1\%]_{FGF10}$, $IC[2\%]_{FGF10}$)
1% and 2% IC gels with 100 ng/mL FGF ($IC[1\%]_{FGF100}$, $IC[2\%]_{FGF100}$)
1% and 2% IC gels with 1000 ng/mL FGF ($IC[1\%]_{FGF1000}$, $IC[2\%]_{FGF1000}$)

As control, bFGF was mixed with 1% simple collagen at concentrations of 10 ($COL[1\%]_{FGF10}$) and 100 ng/mL ($COL[1\%]_{FGF100}$). A cuff-shaped container was filled with these materials containing bFGF and implanted into the rat subfascial space in the same manner. Five rats were used for evaluating each material. The implants were excised 5 days after implantation, and neovascular development in the implants was analyzed.

To assess the effect of bFGF outside of the material, vascular development in the tissue surrounding COL[1%], $COL[1\%]_{FGF100}$, IC[1%] and $IC[1\%]_{FGF100}$ was evaluated. The analysis area was set in the surrounding tissue within 100 μm from the margins of the material, and three parameters of neovascular development were measured in the same manner.

2.7. Statistical analysis

All results are expressed as mean ± standard deviation. Statistical analysis was performed with JMP Statistical Discovery Software ver. 6.0.3 (SAS Institute Inc., Cary, NC). For the results of in vivo experiments, the statistical significance of differences vs. the COL[1%] group was determined by one-way analysis of variance followed by Dunnett's test, and for the results of in vitro experiments, the statistical significance of differences between groups was determined by Tukey's HSD test. Differences were considered statistically significant at p-value < 0.05.

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