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Preparation and characterization of pro-angiogenic gel derived from small intestinal submucosa

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ABSTRACT

Gels derived from decellularized small intestinal submucosa (SIS) have been used to repair ischemic myocardium and deliver protein drug. However, their material properties and effects on cell behavior are not well understood, in part because of the difficulty of gelling *in vitro*. In this study, soluble SIS matrix, which was easily handled and could effectively gel, was successfully prepared using a modified method. Fourier transform infrared spectroscopy confirmed that the SIS gel contained not only collagen but also sulfated glycosaminoglycans (sGAGs). Interestingly, the sustained release of vascular endothelial growth factor and basic fibroblast growth factor within the SIS gel was detected, and no initial burst release was observed. The SIS gel was more capable of evoking neovascularization than collagen type I gel, as determined by tube formation experiments in human umbilical vein endothelial cells, the mouse aortic ring assay, and animal experiments. The upregulated expression of kinase insert domain receptor (KDR), Notch1, and Ang2, the key genes in angiogenesis that were evaluated in HUVECs seeded on the SIS gel, confirmed that angiogenesis bioactive factors contained in the SIS gel are indeed active and effective. The SIS gel significantly promoted neovascularization compared to the collagen type I gel *in vivo*. Histology revealed adequate host tissue response in engraftment both types of gels. Together, these data demonstrate that the SIS gel is a promising and attractive candidate for tissue engineering, especially in promoting vessel formation.

Statement of significance

The material properties of small intestinal submucosa (SIS) gel and the effect of these properties upon cell behavior are not well understood, in part due to the difficulty of gelling *in vitro*. In this study, soluble SIS matrix, which was easily handled and gelled was prepared using modified method. The material properties and biocompatibility of SIS gel were explored. The sustained release of growth factors from this gel was observed along with its degradation *in vitro*. The results demonstrate that the SIS gel promote angiogenesis *in vitro* and *in vivo*. The SIS gel biological properties suggest that the constituent ECM molecules released from the gel remain activity. These findings suggested that the SIS gel was a promising candidate for tissue engineering, especially in promoting vessel formation.

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1. Introduction

The extracellular matrix (ECM) plays an important role in cell growth regulation, survival, maturation/differentiation, and development. ECM biomaterials are usually prepared by decellularization of intact tissues or organs [1,2]. Such biomaterials contain complex ECM components that mimic with the ECM of native tissue, and exhibit attractive bioactivity for tissue remodeling and regeneration [2,3]. They have been used for the repair of a variety of tissues including the lower urinary tract [4], esophagus [5], myocardium [6], and musculotendinous [7] tissues. Decellularized ECM biomaterials can be partially digested by pepsin digestion or high salt protein extraction, solubilized, and polymerized

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in situ to form a gel with porous fibrous nanostructures [8–20]. Compared to the original ECM material, ECM gels with appropriate viscosities can be delivered via minimally invasive surgical techniques with the ability to conform to three-dimensional (3D) spaces after injection. Moreover, their 3D network constituting a complex extracellular environment closely mimics the native tissue [17]. ECM hydrogels retain some of the components and bioactivity of the intact ECM [8,21–24], and their degradation causes the release of many bioactive components, such as peptides, which are useful for cell recruiting [25] and tissue remodeling [26]. ECM hydrogels can deliver cells, drugs, and growth factors, and provide a stable microenvironment for cell growth to facilitate new tissue formation [10,13,19,20,22,27–30]. To date, ECM gels derived from brain [8], bone [9], skin [10], myocardial matrix [11,12], meniscus [13], articular cartilage [14], tendon [15], adipose tissue [16,17], liver [18], bladder [8], and small intestinal submucosa (SIS) [19,20] have been reported.

Among utilized ECM materials, SIS is one of the most comprehensively investigated biomaterial including its structure, composition, biological activity, and the host constructive remodeling response to it. To date, it has been extensively used as scaffolds in tissue engineering and regenerative medicine to organs such as the artery [31], bladder [32], intestinal tract [33], valve [34], esophagus [35], and tendon [36]. The U.S. Food and Drug Administration has approved the use of SIS in humans for urogenital procedures, such as hernia repair, cystoplasties, ureteral reconstructions, stress incontinence, peyronie's disease, penile chordee, and even urethral reconstruction for hypospadias and strictures [37]. SIS could be processed to an injectable form to expand its utility. The SIS gel maintains its structural integrity under physiological conditions, can act as an injectable drug depot [19,20], and produced engineered cardiac tissues with a more physiological contraction rate and higher phenotypic protein expression than Matrigel constructs [21], preserved cardiac function post-myocardial infarction [28]. SIS gel also could serve as a model for dormancy and re-awakening to allow the identification of therapeutic targets for treating micrometastases [38]. Understanding the physical and biological properties of ECM hydrogels may facilitate their more effective utilization for specific therapeutic applications. The physical and structural properties of SIS gels, as well as the release of growth factors from SIS gels are yet to be determined, partially because of the difficulty gelling *in vitro*. SIS gels can be preserved at low temperatures; however, they have a short storage life [39] and are hard to sterilize in liquid form. Thus, for clinical uses, it is best to preserve them by lyophilization to ensure a long shelf life. Therefore, there is a need to optimize the preparation method for SIS gels.

Angiogenesis, the formation of new blood vessels, plays a significant role in tissue development, function, maintenance, repair, and regeneration. In many diseases, such as myocardial and peripheral ischemia, diabetic ulcers, retinal diseases, and chronic wounds, the pathology is caused by a reduced blood supply [40] that results in cell death in the downstream tissue, followed by degradation of the associated ECM. The rapid formation of new blood capillaries is essential for tissue engineering and regenerative medicine by supplying the necessary nutrients and oxygen to and removing waste products from cells. Thus, there is a great need for the development of biomaterials that can encourage angiogenesis. Prior studies have demonstrated conductivity of SIS for myocardial tissue ingrowth and vascularization when applied as an atrial prosthesis and a right ventricular full thickness patch [41,42]. With regard to bioactive properties, SIS promotes proliferation, attachment, and migration of various cell types, and stimulates angiogenesis through an assortment of matrix-embedded angiogenic factors, including connective tissue growth factor (CTGF), basic fibroblast growth factor (bFGF), transforming growth factor-beta (TGF-β), and fibronectin [43]. SIS has also been reported to release growth factors including bFGF, TGF-β1, and VEGF [43–45]. Injectable SIS ECM in an acute myocardial infarction model can increase the numbers of arterioles after injection into myocardium [46]. Previous studies have suggested that the constituent ECM molecules released from the intact scaffold during *in vitro* degradation remained active in the gel [24]. Therefore, we hypothesized that SIS gels may be capable of inducing the angiogenic response favorable to tissue repair. It is very important to confirm the pro-angiogenic ability of SIS gels for its application in tissue engineering and regenerative medicine.

Collagen gels are commonly utilized as a scaffold, with the advantages of being biodegradable and excellent biocompatibility, providing a 3D structure, allowing homogeneous cell distribution and deposition of the ECM, and being able to take any desired shape which adapts to any defect size [47]. Therefore, it was selected as the control material. Based on previous studies of SIS and ECM gels, the objectives of the present study were: (1) to prepare a gel from porcine SIS using a modified method; (2) to characterize the material properties, *in vitro* cell growth characteristics, and *in vivo* host response; and (3) to evaluate the potential of angiogenesis *in vitro* and *in vivo*.

2. Materials and methods

2.1. Preparation of SIS gel

SIS was prepared as previously described [48]. In brief, the porcine small intestine was harvested from healthy home-raised pigs (around 100 kg at 6 months) within 4 h of sacrifice. The original SIS was obtained by mechanical removal of the tunica serosa and tunica muscularis, and then cleaned by continuous washing with a saline solution. The subsequent procedure involved degreasing, enzyme digestion, detergent treatment, lyophilization and sterilization. The SIS was thawed and treated with the following solutions for the aforementioned purposes: a solution containing methanol and chloroform (1:1, v/v) for 12 h, 0.05% trypsin/0.05% ethylenediamine tetraacetic acid at 37 °C for 12 h, and 0.5% sodium dodecyl sulfate (SDS) in 0.9% sodium chloride with continuous shaking on a shaker for 4 h. Finally, the submucous membrane was soaked in 0.1% peroxyacetic acid and 20% ethanol for 30 min, after which the membrane samples were thoroughly rinsed with deionized water to remove residual reagent. All of the samples were freeze-dried under −70 °C with a lyophilizer (CHRIST, GAMMA 2-16 LSC, Germany).

SIS gel was prepared using modified methods (Scheme 1) based on previously published protocols for ECM gels [22]. Lyophilized SIS (Scheme 1A) was powdered using a Wiley Mill (Retsch, MM400, Germany) and filtered through an 80 mesh screen. The comminuted SIS (Scheme 1B) was then enzymatically digested in a solution of 1 mg/ml porcine pepsin (Sigma–Aldrich) in 0.01 M HCl at a constant stir rate (60 rpm) for 48 h at room temperature (25 °C). The final SIS digest solution (Scheme 1C) was freeze-dried again at −70 °C to obtain soluble SIS matrix (Scheme 1D). Then, the soluble SIS matrix was cut into flocculent pieces (Scheme 1E) and sterilized using ethylene oxide (EO) gas. To form a gel, the pieces of soluble SIS matrix were dissolved in phosphate-buffered saline (PBS) and neutralized to a pH of 7.4 with the addition of 0.1 M NaOH. The activity of pepsin was deactivated when the pH was raised to 6 [49]. Then, the neutralized solution (pre-gel) was placed in an incubator heated to 37 °C for 1 h, after which a gel formed (Scheme 1F). SIS pre-gels at concentrations of 20 and 30 mg/ml were prepared. The pre-gel was also injected through a syringe and 18G needle to subjectively determine injectability. Collagen type I gel was evaluated as a control, and was prepared

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