 DOI: 10.1002/masy.200750320

Heparin Immobilized Small Intestinal Submucosa for Cardiovascular Applications

Kyoung Soo Jee,[1] *Jin Woo Bae,*[1] *Jae Seung Shin,*[2] *Dong Keun Han,*[3] *Ki Dong Park**[1]

Summary: A novel chemical modification of biological tissue was developed by the immobilization of heparin to small intestinal submucosa (SIS) using glutaraldehyde (GA). Heparinized SIS tissue was characterized by measuring *in vitro* plasma protein adsorption, platelet adhesion, *in vitro* fibroblast attachment, and *in vivo* calcification. Lower protein adsorption and platelet adhesion were observed on heparinized SIS than non-modified SIS. The attachment of human dermal fibroblast on the heparinized SIS was significantly increased, and the amount of calcium deposited on the modified SIS was much less than fresh SIS. These results demonstrated that heparin immobilized SIS can be applied as a novel bioprosthesis for a variety of cardiovascular applications.

Keywords: bioprosthesis; cardiovascular application; glutaraldehyde; heparin; small intestinal submucosa

Introduction

Since artificial prosthetics such as synthetic polymers and metals were used in the field of cardiovascular surgery, biomaterials possessing essential characteristics of native tissues and organs which are to be replaced have been constantly required for the development of an ideal artificial substitute. Consequently, the use of xenograft and allograft tissue has long been the focus of cardiovascular research.[1] Small intestinal submucosa (SIS), a relatively acellular collagen-based matrix derived from porcine small intestine, has been extensively used as a cardiovascular bioprosthesis such as heart valve,[2] vascular graft,[3] and tissue patch for the repair of myocardial infarction.[4] Collagen-based biomaterial such as SIS has typically required chemical or physical pretreatment to enhance *in vivo* biochemical and mechanical properties. The commonly accepted procedure is to use glutaraldehyde (GA), the crosslinking reagent which can stabilize the collagen-based structure of the tissue.[5,6] The GA fixation accomplishes the low immunogenicity and stabilization of tissue to be implanted. GA treatment has also been associated with several problems, including calcification and cytotoxicity.[7] Heparin binding, which led to the formation of bridge between adjacent fibril, reduced the calcium accumulation site.

In our previous studies, it was also reported that heparinized bovine pericardium was shown to decrease *in vivo* calcification.[5] In this study, SIS was chemically modified with GA and heparin. Heparin immobilized SIS was investigated to study the effect of heparin coupling on blood compatibility, *in vitro* fibroblast attachment, and *in vivo* calcification of SIS.

[1] Department of Molecular Science and Technology, Ajou University, Suwon 443-749, Korea
Fax: (+82) 219 1592
E-mail: kdp@ajou.ac.kr
[2] Dept. of Thoracic and Cardiovascular Surgery, College of Medicine and Cardiovascular Institute of Medical Science Research Center, Korea University, Ansan 425-020, Korea
[3] Biomaterials Research Center, Korea Institute of Science and Technology, P.O. Box 131, Cheongryang, Seoul 130-650, Korea

Experimental Part

Materials

Heparin sodium salt (from porcine intestinal mucosa, 187 unit/mg), bacterial collagenase (Clostridium histolyticum Type II), glutaraldehyde (GA, electron microscopy grade 25%), and sodium borohydride were purchased from Sigma Co. All other reagent grade chemicals and solvents were used as received without further purification.

Methods

Preparation of Heparin Immobilized SIS

Fresh SIS was prepared as previously described.[8] Briefly, freshly harvested porcine jejunum was obtained from a local slaughterhouse. It is inverted and the superficial layers of the tunica mucosa are removed by scrapping with a knife handle. The tissue is then reverted to its original orientation and the serosa and tunica muscularis are removed. The SIS sheets were rinsed extensively in water and sterilized by exposure to 0.1% (v/v) peracetic acid. Finally, the obtained SIS sheets were stored in Hank's balanced solution before use.

The SIS tissue was chemically modified as shown in Figure 1. Heparin was immobilized onto SIS tissue by direct coupling of heparin containing amino groups after GA fixation. A series of control specimens was pretreated with 0.625% (v/v) GA solution for 10 days at 4 °C in PBS (pH 7.4). GA-treated SIS was then incubated in 0.75% (w/v) heparin solution in PBS (pH 11) at 4 °C for 5 days. The modified SIS tissues were rinsed with excess PBS and stabilized by treating them with 0.01N sodium borohydride at 4 °C for 16 h.

Amount of Immobilized Heparin and its Anticoagulant Activity

The amount and anticoagulant activity of heparin coupled to SIS were measured by toluidine blue and Activated Partially Thrombosis Time (APTT) assay, respectively as previously described.[9,10]

In Vitro Protein Adsorption and Platelet Adhesion

In vitro plasma protein adsorption and platelet adhesion test were carried out with the modified SIS tissues (1×1 cm^2) which were equilibrated with PBS (pH 7.4) for 12 h. The total amount of plasma proteins adsorbed onto the specimen was quantitatively analyzed by BCA kit and the number of adhered platelets was counted using hemocytometer.[9]

In Vitro Fibroblast Attachment

Primary human dermal fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) in a humidified environment at 37 °C and 5% CO_2. Cells were seeded at 1×10^3 cell/cm^2 by diluting the cell suspension. After incubation for 3 h at 37 °C in an atmosphere of 5% CO_2, each sample was washed twice with PBS at 37 °C in order to remove nonattached cells and the number of attached cells was assessed in a hexosaminidase reaction.[11]

In Vivo Calcification

In vivo calcification was studied using a rabbit subcutaneous implantation model. GA and heparin treated SIS tissues (1×1 cm^2) were implanted onto the subcutaneous muscle for the period of 8 weeks. After the retrieved tissues were rinsed with distilled water, freeze dried to constant weight, and the amount of calcium

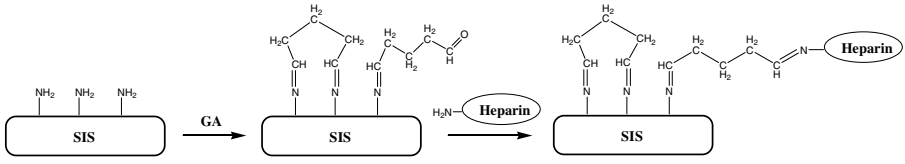

Figure 1.
Schematic diagram of heparin immobilized SIS.

was determined by Inductively Coupled Plasma Atomic Emission Spectrophotometer (ICP, 138 Ultrace, Jobin Yvon Co.) on 1.0 N HCl hydrolysates of dried tissues.

Results and Discussion

Chemical modification of SIS was processed according to the scheme shown in Figure 1. GA was employed as a cross-linking agent to react with amino acid residues of collagen. After GA treatment, heparin was immobilized onto free aldehyde groups of GA already bound to SIS tissue. The chemical reaction between the residual aldehyde and amine groups occurs via Shiff base formation. The unstable Shiff bases were converted to stable secondary amines by $NaBH_4$. The amount and anticoagulant activity of heparin bound to the modified SIS was 0.40 ± 0.08 $\mu g/cm^2$ and 12.7%, respectively.

Figure 2a shows the adsorption of human plasma proteins onto the SIS tissues. The total amount of plasma proteins adsorbed onto SIS-GA-heparin was 1.03 $\mu g/cm^2$, which was lower than that adsorbed onto fresh SIS. The amount of protein adsorbed onto SIS-GA was almost similar to that adsorbed onto SIS-GA-heparin. As shown in Figure 2b, indicating the number of platelets adhered to the modified SIS surface, the adhesion of platelets to SIS-GA-heparin was decreased. These results demonstrated that blood compatibility of the modified SIS was improved due to heparin immobilization.

Additionally, mechanical properties and enzymatic digestion with the modified SIS were investigated. Durability and resistance to collagenase digestion of heparin immobilized SIS was similar to GA-treated one.

In vitro human dermal fibroblasts attachment on SIS-GA-heparin was significantly increased in comparison with fresh SIS (Figure 3). Several cell culture studies demonstrated that SIS construct as a bioscaffold, including various growth factors and ECM proteins, can promote cell growth.[12] Heparin has been known to regulate cellular behaviors by binding with a number of biologically important proteins such as growth factors and ECM proteins.[13] Therefore, this result supported that heparin immobilized SIS can improve the attachment and proliferation of seeded cells. Also, this modified SIS can be reduced its cytotoxicity by removing the residual aldehyde groups, which are resulted from GA fixation.

From *in vivo* calcification, calcium contents of retrieved tissues are summarized in Table 1. When compared with the GA treated SIS, calcium deposition was significantly decreased in heparin immobilized SIS.

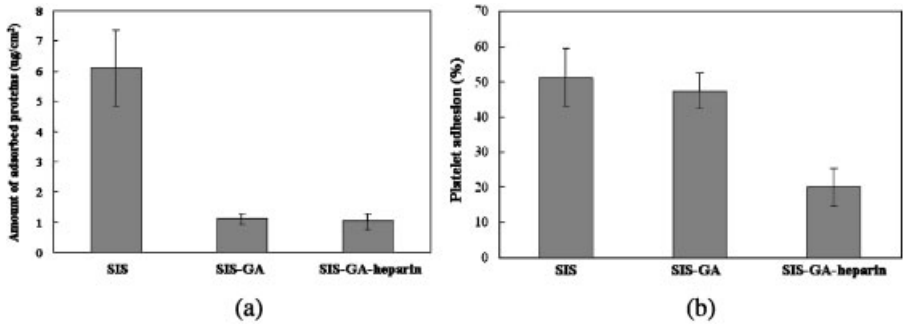

Figure 2.
In vitro blood compatibility of the modified SIS tissues. Plasma protein adsorption (a) and platelet adhesion (b) after 1 h incubation. (Mean $\pm$ SD, n = 4)

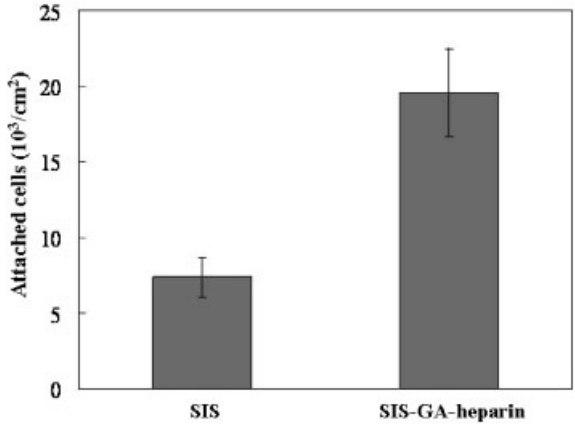

Figure 3.
In vitro attachment of human dermal fibroblast on the modified SIS after 3 h incubation. (Mean ± SD, n = 4)

Table 1.
In vivo calcium deposition on the modified SIS.

Sample	Ca	P
SIS-GA	194.8 ± 14.7	14.3 ± 0.9
SIS-GA-heparin	84.6 ± 9.7	2.9 ± 0.3

Unit: μg/mg of tissue (Mean ± SD, n = 4).

Residual aldehyde groups after GA treatment can be further reacted with amine groups of materials. It may be hypothesized that, after GA treatment, free aldehyde moieties on the surface of the implant undergo oxidation, followed by acid formation, and this acid probably traps the host plasma calcium. The slow release of GA from the prosthesis promotes the host-plasma calcium-acid bound complex.[14] Immobilization of heparin onto SIS tissue may fill the intertropocollagen spaces, blocks the potential binding sites, and thus makes the decreased calcium deposition.

Conclusions

A novel heparinized SIS was prepared by coupling heparin after GA treatment. Heparin immobilized SIS was evaluated *in vitro* and *in vivo* to investigate the effect of modification. Based upon the obtained results, heparin immobilized SIS may be useful for the development of calcification-resistant and biocompatible tissue patches, vascular graft, and heart valve for cardiovascular applications.

Acknowledgements: This work was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare (A050082) and Nano-Biotechnology Project (Regenomics), Ministry of Science & Technology (B020214), Republic of Korea.

[1] S. L. Hilbert, V. J. Ferrans, M. Jones, *Med. Prog. Technol.* **1988**, *14*, 115.
[2] R. G. Matheny, M. L. Hutchison, P. E. Dryden, M. D. Hiles, C. J. Shaar, *J. Heart Valve Dis.* **2000**, *9*, 769.
[3] R. Roeder, J. Wolfe, N. Lianakis, T. Hinson, L. A. Geddes, J. Obermiller, *J. Biomed. Mater. Res.* **1999**, *47*, 65.
[4] S. Badylak, J. Obermiller, L. Geddes, R. Matheny, *The Heart Surgery Forum* **2003**, *6*(2), E20.
[5] W. K. Lee, K. D. Park, D. K. Han, H. Suh, J. C. Park, Y. H. Kim, *Biomaterials* **2000**, *21*, 2323.
[6] K. S. Jee, Y. S. Kim, K. D. Park, Y. H. Kim, *Biomaterials* **2003**, *24*, 3409.
[7] C. E. Schmidt, J. M. Baier, *Biomaterials* **2000**, *21*, 2215.
[8] K. Lindberg, S. Badylak, *Burns* **2001**, *27*, 254.
[9] K. S. Jee, H. D. Park, K. D. Park, Y. H. Kim, J. W. Shin, *Biomacromolecules* **2004**, *5*, 1877.
[10] H. D. Park, W. K. Lee, T. Ooya, K. D. Park, Y. H. Kim, N. Yui, *J. Biomed. Mater. Res.* **2002**, *60*, 186.
[11] M. V. Erokhina, A. A. Shtil, S. S. Shushanov, T. A. Sidorova, A. A. Stavrovskaya, *FEBS Lett.* **1994**, *341*, 295.
[12] S. Badylak, *Transplant Immunology* **2004**, *12*, 367.
[13] I. Capila, R. J. Linhardt, *Angew. Chem. Int. Ed.* **2002**, *41*, 390.
[14] J. Chanda, *Ann. Thorac. Surg.* **1995**, *60*, S339.

