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Immobilization of RGD Peptides onto Decellularized Valve Scaffolds to Promote Cell Adhesion

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Abstract: Porcine aortic valves were decellularized with trypsinase/EDTA and Triton-100. With the help of a coupling reagent Sulfo-LC-SPDP, the biological valve scaffolds were immobilized with one of RGD (arginine-glycine-aspartic acid) containing peptides, called GRGDSPC peptide. Myofibroblasts harvested from rats were seeded onto them. Based on the spectra of X-ray photoelectron spectroscopy, we could find conjugation of GRGDSPC peptide and the scaffolds. Cell count by both microscopy and MTT assay showed that myofibroblasts were easier to adhere to the modified scaffolds. It is proved that it is feasible to immobilize RGD peptides onto decellularized valve scaffolds, and effective to promote cell adhesion, which is beneficial for constructing tissue engineering heart valves in vitro.

Key words: RGD peptide; decellularized valve scaffold; cell adhesion; tissue engineering heart valve

1 Introduction

The evolving field of cardiovascular tissue engineering utilizes a variety of scaffold materials and autologous cells seeding[1]. In comparison with synthetic polymers, the use of xenogeneic decellularized valve scaffolds is more hopeful for the clinical studies of tissue engineering heart valve (TEHV). It has been demonstrated by experiments in vitro that this type of scaffolds are mainly made up of collagen with complicated structure, and the decellularization process could put effects on host cells infiltration and proliferation, maybe resulted from incomplete extracellular matrix (ECM) preservation[2]. It is a new challenge to control biocompatibility of decellularized valve scaffolds for construction of TEHV. One approach is to control cell adhesive properties by grafting surface modification, in order to promote ingrowth of host cells onto surface or into porous structure of these scaffolds.

Since RGD peptides (R: arginine; G: glycine; D: aspartic acid) have been found to promote cell adhesion in 1984, numerous materials have been RGD functionalized for academic studies or medical applications[3,4]. It has been shown that attachment activity of collagen, either monomer alone or in combination with other ECM proteins, is at least in part dependant on the sequence of RGD containing peptides[5]. The peptides specifically and effectively improve the cell-matrix attachment by binding to integrins, which are a large family of cell surface receptors[6]. And Data have been shown that adhesive properties of collagen can be modified by covalent linkage with these peptides, such as containing arginine -glycine -aspartic acid-serine (RGDS) sequences[5].

The goal of this work was to control adhesive properties of decellularized valve scaffolds by surface modification of RGD peptides. In this paper, one method for immobilization of RGDS containing peptides with decellularized valve scaffolds was described. And data about cell adhesion after this modification were also presented.

2 Experimental

2.1 Porcine valves decellularization

Porcine hearts were obtained under clean conditions. Aortic valves were excised and washed in sterile phosphate-buffered saline (PBS) solution followed by an overnight incubation at 4 ℃ in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics. Then the valve leaflets were placed in a solution of 0.05% trypsinase and 0.02% EDTA at 37 ℃ for 12 h. After a washing step, they were placed in a solution of 1% detergent TritonX-100 at 4 ℃ for 48 h. At last, they were placed in a solution of 20 μg/mL RNase and 200 μg/mL Dnase

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at 37 ℃ for 1 h. These scaffolds were rapidly kept in deep cold refrigerator at –80 ℃, and prepared to use by thawing slowly at room temperature[2].

2.2 Cell culture

Under aseptic conditions, thoracic aorta of rats was excised and cut out to tissue pieces. They were put into a culture bottle and incubated in DMEM supplement with 10% fetal bovine serum in the condition of 37 ℃ and 5% CO_2. Fibroblasts and smooth muscle cells were isolated 5 to 7 days later, fused and grew up 3 to 4 weeks later. After identification, the cells were ready for use after 4 to 5 passages[7].

2.3 Immobilization of RGD peptides onto decellularized valve scaffolds

Glycine-arginine-glycine-aspartic acid-serine -proline -cysteine peptide (GRGDSPC) was synthesized by GL Biochem Company (Shanghai, China) with method of Fmoc solid phase. It was described as NH_2-Gly-Arg-Gly-Asp-Ser-Pro-Cys-COOH with acetate end protected. Before froze-drying into powder, it was purified and identified by means of mass spectrum (MS) and high performance liquid chromatography (HPLC).

After GRGDSPC peptide was solved by double distilled water at a concentration of 1 mg/mL, decellularized valve scaffolds were coupled with it via a heterobifunctional coupling reagent, Sulfosuccinimidyl-6-(3'-(2-PyridylDithio)-Propionamido)-hexanoate (Sulfo-LC-SPDP) (Pierce Biochem, USA). The chemical reaction were carried out in PBS buffer (pH=5) for 48-72 h at room temperature under constant stirring[5].The valve scaffolds immobilized with GRGDSPC peptide were regarded as group A (n=6). Meanwhile, the scaffolds simply mixed with peptide were regarded as group B, and untreated ones were group C. Three groups were dried and vacuumized at 4 ℃. X-ray photoelectron spectroscopy (XPS) was used for surface analysis with an apparatus of ESCALab MK2.

2.4 Adhesive experiments

Three groups of scaffolds were put into different wells in a 24-well tissue culture plate. Myofibroblasts were made in cell suspension and counted as Sum one (S_1). Then 0.5 mL cell culture media were added at a concentration of 5×10^5 cells/cm^2 and incubated at 37 ℃, 5% CO_2 for 2 h. Non-adherent cells were washed by a 0.5 mL cell-free culture media and landed on bottom of the plate, which were directly counted microscopically as Sum two (S_2). Adherent cells were released with 0.25% trypsinase solution and counted as Sum three (S_3).

Another method for adhesion assay is called modified 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Number of cells is directly proportional to the absorbency (OD) level by a simple colorimetric assay at a wavelength of 490 nm. We modified this assay to count non-adherent cells for decellularized valve scaffolds After depicting a standard curve for S_2 and OD, the absorbency of

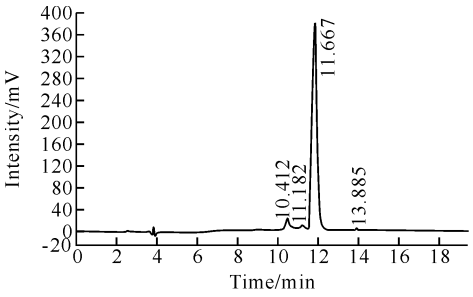

Fig.1 Results of chromatogram for identification of GRGDSPC peptide

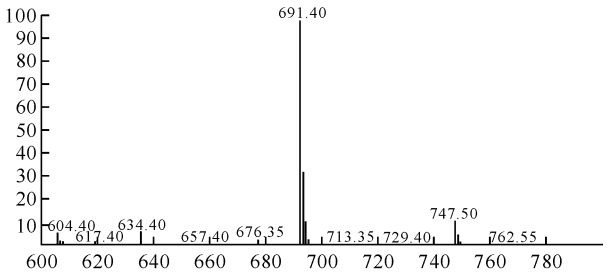

Fig.2 Results of mass spectrum for identification of GRGDSPC peptide

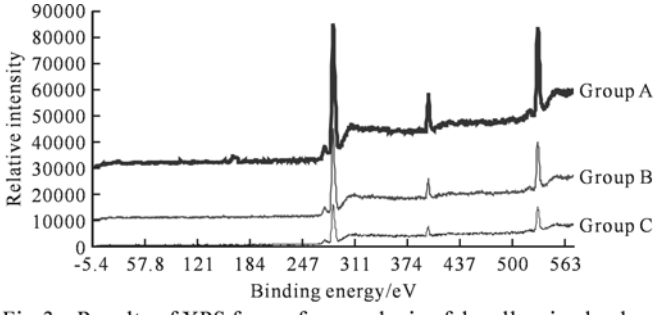

Fig.3 Results of XPS for surface analysis of decelluarized valve scaffolds. It shows that GRGDSPC peptide was grafted on scaffolds of group A by chemical bonds

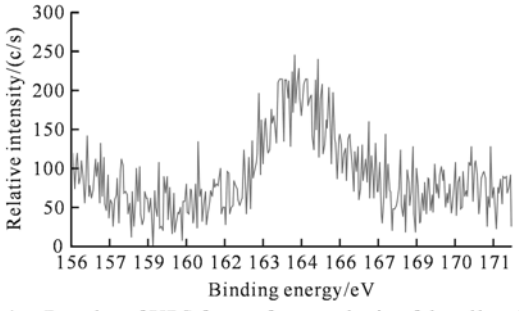

Fig.4 Results of XPS for surface analysis of decelluarized valve scaffolds. The sulfur element and disulfide bond was found on scaffolds of group A in the binding energy range of 163.1-165.7 eV

different scaffolds for a variety of cell attaching time (1, 2, 6, 4, 8 h) and peptide concentrations (0.2 mg/mL, 1 mg/mL, 5 mg/mL) for 2 h attaching were measured[7].

2.5 Morphological observations

One specimen of groups after cell attachment for 12 h was fixed at room temperature and stained with standard hematoxylin-eosin (HE). Images were analyzed by light microscopy (Olympus BM200, Japan). Another specimen was fixed, dehydrated and sputtered with gold/palladium. Images were viewed by a Hitachis-520 (Japan) scanning electron microscope (SEM)(1000-3000 magnification).

2.6 Statistics

All values are shown as means±SD. Significance difference among groups were estimated using software package SPSS for windows version 13.0. *P* value less than 0.05 were considered significant.

3 Results and Discussion

3.1 Decellularization protocol and cell culture

The results from histology, type I collagen immunochemistry and cell DNA content assay, showed that the decelluarized valve scaffolds produced by trypsinase/EDTA combined with detergent Triton-100 were decellularized effectively and their ECM (especially collagen fibers) were nearly intact. Results of immunocytochemistry with labelling antibodies against α-smooth muscle actin and desmin, and transmission electron microscopy (TEM) indicated that the rat aortic cells isolated and expanded in our laboratory was characterized as myofibroblasts.

It is well-known that the decellularized valve scaffolds are promising scaffold material for TEHV due to their excellent tissue compatibilities and bionic mechanical properties superior to any synthetic polymers. Previous living cells and soluble protein of fresh porcine aortic valves were almost completely eliminated with decellularization protocols of trypsinase, EDTA and detergent[2]. The remnant three-dimensional structure of this type of scaffolds is similar to natural valve and adapts to homogenic cells for localization, migration, growth and differentiation. Our former works demonstrated impaired cell adhesive properties of the scaffolds resulted from partly loss of ECM proteins, such as soluble collagen and proteoglycan molecule. Therefore it is necessary to enhance cell adhesion to this type of scaffolds, and surface modification is a good choice and a brand new research field[8].

3.2 Peptide identification and scaffolds surface analysis

HPLC and MS analyses demonstrate that the purity of the GRGDSPC peptide obtained by solid phase synthesis revealed degree of higher than 95% and molecular weight in the range of 684-691 kDa (Figs.1 and 2). Based on the spectra of XPS, no sulfur element was found on decellularized valve scaffolds in group B and C. But sulfur element and disulfide bond was found on scaffolds in group A in the binding energy range of 163.1-165.7 eV. This result showed that GRGDSPC peptide in group A was grafted on the disposed collagen fibers of scaffolds by chemical bonds. This means that it was feasible and effective for RGD peptides to be immobilized onto decellularized valve scaffolds with help of Sulfo-LC-SPDP (Figs.3 and 4).

It is well-known that RGD peptides are one of the most effective and widely used peptides to promote cell adhesion. As an important sequence among fibronectin and laminin, the short peptides can enhance the cell-matrix attachment by binding to cell surface receptors, called as integrins. Surface modification of synthetic RGD containing peptides would not change physical structure and chemical characteristics of materials and thus can be controlled more easily than other adhesive alternatives[9]. The attachment activities of many kinds of polymer (like PGA, PLGA) and biological materials (like fibrin) have been verified to be enhanced by immobilization with RGD peptides for tissue engineering[10,11]. Data from literatures have shown

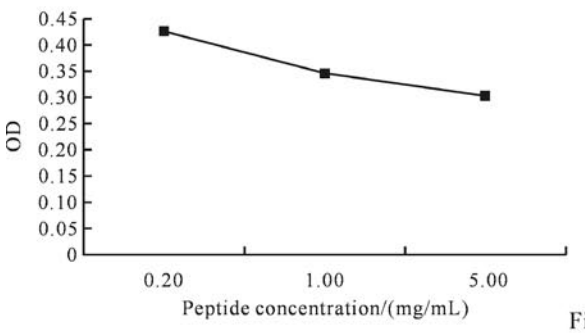

Fig. 5 Chart of relationship between OD means and peptide concentrations

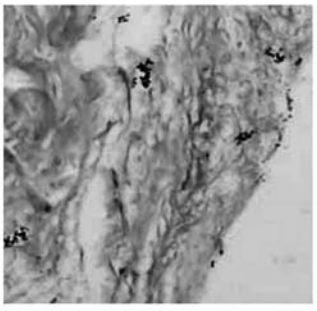

Fig.6 Histological pictures by HE staining of group A after 12 h attachment (×200)

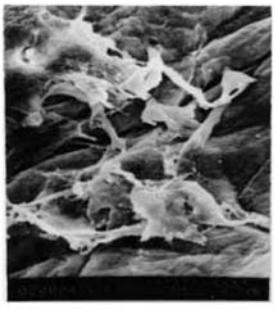

Fig. 7 SEM images of group A after 12 h attachment (×1100)

Table 1 Results of cell adhesion assays for decelluarized valve scaffolds of groups

	Group A	Group B	Group C
S_3 with sedimentation method ($\times 10^4$cells/mL)	17.25±5.188*	12.25±4.349	7.75±1.500
OD with MTT assay	0.592±0.074#	0.826±0.088	0.890±0.014

* *P* values of group A compared to group C<0.05; # *P* values of group A compared to group B and C<0.05

Table 2 Results of MTT assays of decelluarized valve scaffolds for different cell attachment time

	Attach 1 h	Attach 2 h	Attach 4 h	Attach 6 h	Attach 8 h
Group A	0.694±0.049#○	0.592±0.074#⊿	0.410±0.158#	0.361±0.056#	0.281±0.140#
Group B	0.840±0.085○	0.826±0.088⊿	0.498±0.217	0.611±0.064	0.556±0.021
Group C	0.815±0.007○	0.890±0.014⊿	0.503±0.158	0.668±0.074	0.506±0.107

P values of group A compared to group C<0.05; ○ *P* values of group A attach 1 h compared to 4 h,6 h,8 h<0.05;
⊿ *P* values of group A attach 2 h compared to 4 h,6 h,8 h<0.05

that adhesive properties of collagen could be modified by covalent linkage with RGDS containing peptides. GRGDSPC peptide used in this paper not only contains RGDS sequence, which could promote cell adhesion, but also has cysteine residue at the carboxyl-terminus, which is helpful for structure analysis.

There are two ways for surface modification. Compared to simple adsorption of RGD peptides which leads to unstable anchoring and poor cell attachment, chemical coupling method via a covalent bond is more stable and less unwanted plasma protein deposition. This is also proved, by better adhesion-enhancing effects on scaffolds in group A than in group B in this work. SPDP was chosen as the coupling reagent because it contains N-hydroxyl succinimide and 2-pyridyl disulfide group, which are able to react specifically with primary amines present on the decelluarized valve scaffolds and sulfhydryl groups on the GRGDSPC peptide, respectively. Since the two reactive groups are directed toward different functional groups and because the scaffolds do not contain sulfhydryl groups (cysteine residue), the conjugation could be conducted in separated sequential steps with little chance for the introduction of subsidiary reactions. Also, by using a heterofucntional reagent, intermolecular couplings are easily introduced without concomitant formation of intra-molecular coupling[5,8].

Sulfo-LC-SPDP is a modified reagent in the family of SPDP. It is water-soluble and can take part in the reactions as above at room temperature under stirring conditions. From the spectra of XPS, we demonstrated that the scaffolds themselves did not contain sulfur element as the results of group C, simple mixture could not bind scaffolds and peptide chemically as that in group B, and the peptide could be immobilized on the scaffolds successfully as group A. It was shown that coupling with Sulfo-LC-SPDP is a good and convenient approach for decelluarized valve scaffolds immobilized with RGD peptides[5,8].

3.3 Adhesive experiments

With method of the sedimentation, S_1 theoretically, equals to S_2+S_3. S_3 after 2 h attachment in group A was $(17.25\pm5.188)\times10^4$ cells/cm^2 and significantly more than that in group C (P<0.05) (Table 1). Through the standard curve of MTT assay in a 24-well tissue culture plate, there was a linear regression relationship between S_2 and OD with relative coefficient of 0.940. With method of modified MTT assays, OD of non-adherent cells on scaffolds in group A was significantly less than on that in group C (P<0.05) (Table 1). Both results demonstrated that host cells were more prone to attach decelluarized valve scaffolds after GRGDSPC peptide immobilization.

During cell attachment time within 8 hours, OD of non-adherent cells on scaffolds in group A was significantly less than that in group B and C (P<0.05) (Table 2). Moreover, OD of group A, B and C for cell attachment 1 h and 2 h were significantly less than that for 4, 6 and 8 h (P<0.05) (Table 2). For GRGDSPC peptide immobilized scaffolds in group A at different concentrations (0.2,1,5 mg/mL) for 2 h attachment, there was a linear negative regression relationship between OD of non-adherent cells and peptide concentration with relative coefficient of -0.822 (Fig.5).

Aortic myofibroblast is one of the most widely used seed cells for TEHV, whose biological characteristics are like valve interstitial cells. Valve cell adhesion, usually taking place at the first 12 hours

after cell-material contact, is a process composed of cell attachment, spreading, cytoskeleton adjustment and focal adhesion kinase activation, and is also the prerequisite of cell growth, differentiation, migration and ECM proliferation[9,12]. In this work, adherent-cells of scaffolds were counted as S_3 with sedimentation method. Although S_3 directly referred to cell attach amounts, it was not reliable for bad controls of digest time and inaccurate optical count. MTT assay could quantitate cell amounts on the plate reliably[7]. After the linear relationship between OD and S_2 was made, this assay was modified and used for non-adherent cell quantity. Results of MTT assay and sedimentation method both showed that cells were more prone to attach decelluarized valve scaffolds after RGD peptide immobilization, as in group A. And within 8 hours, the degree of cell attachments increased progressively along with attaching time. Meanwhile there was also a linear relationship for GRGDSPC peptide immobilization at different concentrations.

3.4 Morphological observations

By optical microscopy, myofibrobalsts of group A were found to disperse or distribute like small dumplings (Fig.6). More cells adhered to decellularized valve scaffolds in group A according to SEM image. And the adherent cells grew up well in the shape of shuttle or roundness. The original three dimensional structure of decellularized valve scaffolds was very clear (Fig.7). The morphological observations demonstrated similar results as the 3.3. adhesion assays.

4 Conclusion

In a conclusion, it is the first report on decelluarized valve scaffolds immobilized with RGD peptides. The experiments preliminarily proved the feasibility of this type of surface modification and enhancing effects on cell adhesive properties of the scaffolds. It can be anticipated that the construction of TEHV with the help of RGD-immobilized scaffolds would be facilitated owing to the promotion of seed cell infiltration and proliferation. The accurate control of reaction dosages of peptides and decellularized valve scaffolds and molecular mechanisms between them are still waiting for next study.

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