Covalent linkage of heparin provides a stable anti-coagulation surface of decellularized porcine arteries

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Abstract

Establishing thrombosis-resistant surface is crucial to develop tissue-engineered small diameter vascular grafts for arterial reconstructive procedures. The objective of this study was to evaluate the stability and anti-coagulation properties of heparin covalently linked to decellularized porcine carotid arteries. Cellular components of porcine carotid arteries were completely removed with chemical and physical means. Heparin was covalently linked to the decellularized vessels by a chemical reaction of the carboxyl end of amino acids with hydroxylamine sulphate salt and heparin-EDC. Bound heparin contents were measured by quantitative colorimetric assay of toluidine blue staining. The average content of heparin in treated vessels was 35.6 ± 11.6 mg/cm² tissue, which represented 6.21 ± 2.03 UPS heparin/cm² tissue. The stability of heparin linkage was tested by incubating the heparin-linked vessels either in PBS at 37°C or in 70% alcohol at room temperature up to 21 days, showing no significant reduction of heparin content. Anti-coagulation property of bound heparin was determined with a clotting time assay using fresh dog blood. Standardized small pieces of non-heparin-bound vessels were clotted in fresh dog blood within 10 min., whereas all heparin-bound vessels did not form clot during 1-hr observation. In vivo platelet deposition of the vessel was determined with a baboon model of the femoral arteriovenous external shunt and 111Indium labelling of platelets. There were 1.38 ± 0.07 × 10⁹ and 0.64 ± 0.11×10⁹ baboon platelets deposited on the control and heparin-linked vessels, respectively, at 60 min. These data demonstrate that covalent linkage of heparin provides an effective and stable anti-coagulation surface of decellularized porcine carotid arteries. This study may suggest a new strategy to develop tissue-engineered biological vascular grafts, which could be used for human coronary or low extremity artery bypasses.

Keywords: vascular graft • heparin • decellularization • porcine artery • vascular tissue engineering

Introduction

More than half a million coronary artery bypass grafts are implanted each year, creating a great demand for small-calibre vascular grafts. Autologous saphenous vein graft is widely regarded as the ideal bypass conduit in small-diameter (<5 mm) bypass procedures including coronary and infragenou arterial revascularization. However, nearly 30% of patients do not have a suitable saphenous vein due to size mismatch, previous procedures or venous disease [1, 2]. In addition, the 4-year patency rate of saphenous vein is about 40–70% [3]. It would be desirable to have an alternative vascular prosthesis available for small-calibre reconstruction with similar or better patency rates compared with the autologous saphenous vein graft.

Although the synthetic vascular prostheses, such as Dacron fabric grafts and expanded polytetrafluoroethylene (ePTFE), perform well in large-vessel reconstructions, these materials are not suitable for small-calibre arterial reconstructions [4, 5]. Primary patency rates with ePTFE at 4 years for infrapopliteal and aorta-coronary bypass, for instance, are only 12% and 14%, respectively [6–8]. The poor clinical outcome of prosthetic grafts in arterial reconstruction is mainly due to early thrombosis [6–8]. Much effort has therefore been devoted to improve graft patency by surface modification with anti-thrombotic molecules. One pharmacologic agent that has received wide research focus as a means to reduce the thrombogenicity of the graft is heparin. Heparin is a major anti-coagulant with an activity mediated primarily through its interaction with antithrombin, and it has been shown to prevent thrombus formation [9]. In a recent clinical trial, however, no measurable effect of heparin immobilization on
systemic markers of haemostasis was found using a heparin-bound ePTFE graft in vivo [10].

Due to the low success rates with synthetic materials in small-calibre vascular grafts, attempts have also been made at using biological grafts such as decellularized porcine carotid arteries for vascular prostheses since these acellular tissues could retain natural mechanical properties and promote remodelling of the prosthesis by neovascularization and recellularization by the host [11]. However, little was known about the anti-thrombosis property of the heparin-coated decellularized vessels.

The objective of this study was to evaluate the stability and anticoagulation properties of heparin covalently linked to decellularized porcine carotid arteries. We hypothesized that the coating of the decellularized porcine arteries with heparin would provide a non-thrombogenic surface for the graft, which would reduce platelet deposition in the xenograft. This study may suggest a new strategy to develop tissue-engineered biological vascular grafts, which could be used for human coronary or low extremity artery bypasses.

**Methods**

**Chemicals and reagents**

All solutions were made with deionized water unless otherwise noted. Tris-HCl was obtained from Invitrogen Co. (Carlsbad, CA, USA). Phosphate buffered saline (PBS) solution, hydroxylamine sulphate salt, 1-ethyl-3(3-dimethylaminoporpyl) carbodiimide (EDC), heparin sodium salt and toluidine blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

**Decellularization of porcine carotid artery**

Porcine common carotid arteries were harvested from a local slaughterhouse. Animals ranged in weight from 160 kg to 220 kg and in age from 1 to 2 years. Animals were killed by exsanguination through a puncture wound in the neck. Within about 15 min. after death, common carotid arteries were dissected from a ventral midline neck incision using careful sharp dissection technique and harvested in 15–20 cm long. Vessels were immediately rinsed in ice cold PBS to remove any residual blood clot and then stored in 50-ml centrifuge tubes in PBS on ice until returned to the laboratory. Vessels were decellularized using a complex process developed in our laboratory involving hypotonic breakdown, multiple enzymatic digestions and detergent treatments, and mechanical means [11]. Characterizations of the decellularized porcine carotid arteries were described in our previous publication [11]. Briefly, both histochemistry and transmission electron microscopy analyses confirmed that the cellular components such as cell membranes and nucleus were successfully removed from the arteries, whereas extracellular matrix scaffold remained intact.

**Covalent linkage of heparin**

For heparin immobilization, decellularized vessels were pre-treated by incubation in 1 M hydroxylamine sulphate salt at room temperature on an orbital shaker for 12 hrs. Vessels were rinsed in distilled water and next immersed into heparin-EDC solution (1.67 g EDC + 0.835 g heparin sodium salt + 200 ml 0.05 M HCl) with a pH of 1.5 for 48 hrs on an orbital shaker (Fig. 1A). Following heparin immobilization, vessels were rinsed in distilled water and stored in 70% alcohol at 4°C.

**Determination of heparin content**

A colorimetric assay of the toluidine blue staining was used to determine the amount of heparin in a sample [14]. This method is one of the most commonly used methods to detect the contents of immobilized heparin. In preparing a calibration curve, 2.5 ml of toluidine blue 0 was taken into each of seven different test tubes. Varying amounts of standard heparin ranging from 0.01 to 0.07 mg were added to the dye and the volume made up to 5 ml by adding 0.2% sodium chloride solution. The test tubes were agitated in a vortex for 30 sec. A 5-ml solution of n-hexane was added to each tube and vortexed for 30 sec. The aqueous layer of all samples was diluted 1:10 with ethanol. The optical density of each sample was determined spectrophotometrically at 631 nm within 30 min. of reaction. The heparin-linked porcine arteries were analysed for heparin by placing a 1 x 1 cm segment into a test tube and adding 2.5 ml of toluidine blue 0 followed by 2.5 ml of 0.2% sodium chloride solution. The remaining procedure was the same as for the soluble heparin assay. The heparin content (µg/cm² tissue) was calculated based on the standard curve and converted to UPS/cm² (heparin activity unit) based on heparin contents and the activity of heparin used.

**Clotting time**

Vessels were cut open longitudinally and 1 cm³ pieces of vessels were cut from the middle of each. The adventitia was gently removed from each sample by gentle dissection. Each 1 cm³ square tissue sample was then cut into nine equal size pieces and placed into 5-ml glass tubes. Venous blood was drawn from a healthy dog and subsequently 2 ml of blood was quickly placed into each glass tube with the tissue samples. Tubes were then quickly covered with paraffilm and placed on a sample rocker. The tubes were visually inspected for clot formation on the tissue samples as they were rocked. The elapsed time between when the blood was applied to the sample and the formation of clot was recorded as the clotting time. Tests were stopped after 1 hr if no clot was formed. The dog blood was used for the clotting time experiment because the dog model of graft implantation will be used for evaluation of our heparin covalently linked decellularized porcine carotid artery grafts. Human blood for the clotting time experiment was performed and reported in our previous publication [11].

**Platelet deposition in baboon external shunt model**

Thrombogenicity of heparin-linked decellularized porcine carotid arteries was analysed with exteriorized arteriovenous (AV) shunts in the baboon model, which is well established and previously described [15–18]. We have resources and extensive experience in utilizing this model to generate
reliable data about graft thrombogenicity. This non-human primate model offers the advantages of similar vascular anatomy and cellular characteristics compared with humans. This model simulates acute thrombosis with native or anti-coagulated blood, and a testing graft for thrombogenicity is inserted as an extension piece into a non-thrombogenic chronic baboon femoral AV shunt. The shunts with heparin-linked vessels were constructed. Five-cm-long heparin-linked vessel was connected to two segments of silicon tubings (0.25-in. diameter) by super glue and a Teflon mandrel. After super glue had sufficiently dried, silicon glue was applied to completely cover the vessel and the tubing in close proximity. Before the silicon glue was allowed to dry, another larger silicon tube was applied over the vessel to provide a bridge to the two segments of silicon tubing for stability. The constructs were allowed to dry overnight completely. The mandrels were then gently slid from the inside of the construct and stored in 70% ethanol. Pressure testing was conducted to evaluate shunt performance at and above physiological pressure. Pressure tests began at atmospheric pressure and reached 250 mm Hg. The shunt-vessel device was connected to chronic AV shunt, which was composed of silicone rubber tubing (3-mm ID, Dow Corning) placed between the baboon femoral artery and vein. Four juvenile male baboons (Papio anubis/cynocephalus) weighing 9–12 kg were studied. Platelet deposition was measured with $^{111}$In-labelled platelets as previously described [17, 18]. In brief, autologous baboon platelets were labelled with 1 mCi of $^{111}$In-oxine. The accumulation of $^{111}$In-labelled platelets was measured continuously with a gamma scintillation camera (General Electric 400T, General Electric Co., Niskayuna, NY, USA). The AV shunt was maintained at arterial flow rates (100 ml/min.) for 60 min. without systemic anti-coagulation. Data were stored at 5-min. intervals and analysed with a computer-assisted image-processing system interfaced with the camera. The total number of deposited platelets was calculated by dividing the deposited platelet radioactivity (counts per minute) by the whole-blood $^{111}$In-platelet activity (counts per min/ml) and multiplying by the circulating platelet count (platelets per millilitre) [15, 16]. Four control grafts and heparin-linked grafts were tested. Animals were housed and cared for in the animal facilities of the Emory University Yerkes Primate Center with approved protocols and compliance with the standards in 'The Guide for the Care and Use of Laboratory Animals'.

Statistical analysis

Differences between groups were tested using the analysis of variance (ANOVA) test with significance considered to be $P < 0.05$. Results are reported as mean ± standard error.

Results

Efficiency and stability of heparin-covalent linkage

More than 300 decellularized pig carotid arteries were covalently linked with heparin. Heparin-linkage in the vascular tissues was also directly visualized with toluidine blue staining of tissue section (5 μm thickness), and blue colour indicates positive staining (Fig. 1B). Decellularized vessels with heparin linkage showed a strong toluidine blue staining at the luminal surface and weak staining at the media and adventitia layers of the vessel (Fig. 1B), whereas decellularized vessels without heparin treatment did not stain with toluidine blue (Fig. 1C). These results indicated that heparin was successfully linked to the decellularized vessels. Through a quantitative analysis, the average content of heparin in treated vessels was $35.6 ± 11.6 \mu g$ heparin/cm$^2$ tissue (ranging from 31 to 42 μg heparin/cm$^2$ tissue), which represented $6.21 ± 2.03$ UPS heparin/cm$^2$ tissue (ranging from 5 to 8 UPS/cm$^2$ tissue).
The stability of heparin linkage was tested by incubating the heparin-linked vessels either in PBS at 37°C or in 70% alcohol at room temperature up to 21 days (Fig. 2). Aseptic conditions were used in the procedure described. Heparin contents after culture did not show a significant reduction in both PBS and 70% alcohol. Furthermore, several decellularized-heparin-linked vessels were stored in 70% alcohol for more than 2 years and the contents of heparin-covalent linkage were not reduced. Thus, the heparin immobilization procedure is effective and reliable, and heparin linkage is stable. Furthermore, the processed vessels are easily sterilized by 70% alcohol and enable to simple long-term storage.

Clotting time of heparin-linked porcine arteries in dog blood

A functional clotting time assay was performed to verify the functionality of the bound heparin in vessels. All control vessels formed clots within 10 min. after mixed with fresh dog blood (Fig. 3), which was comparable to the clotting time (13 min.) in fresh human blood [11]. However, heparin-linked vessels did not form any clots after 60 min. mixing with dog blood (Fig. 3), which was consistent with the results of human blood [11]. Thus, bound heparin possesses strong anti-coagulation function.

Platelet deposition to heparin-linked porcine arteries in the baboon external shunt model

Heparin-linked vessels and control vessels (5 cm long) were mounted inside of the silicon tubing, which was attached to femoral arteriovenous conduits of male baboons for 60 min. The autologous platelets of baboons were pre-labelled with 111Indium. Platelet deposition was measured by scintillation camera imaging analysis (Fig. 4A). The heparin-coated vessels showed a significant less platelet deposition compared with control vessels (Fig. 4B). At 60 min., heparin linkage substantially inhibited platelet deposition by 54% compared with controls in baboon blood (n = 4, P < 0.05, Fig. 4B).

Discussion

The current study demonstrates that the covalent linkage of heparin to the decellularized graft is a new strategy to provide an effective and stable anti-coagulation surface of the graft. Specifically, heparin was covalently linked to the decellularized vessels by a chemical reaction of the carboxyl end of amino acids with hydroxylamine sulphate salt and heparin-EDC. Bound heparin contents and stability of the graft were confirmed by the chemical quantitation method. Anti-coagulation property of bound heparin was determined by both in vitro clotting time test and ex vivo baboon model of quantitative platelet deposition assay. These new findings significantly advance the development of tissue-engineered biological vascular grafts, which could be used for human coronary or low extremity artery bypasses.

The rationale to remove the cellular components from the vascular tissues is to develop a non-immunogenic vascular graft from porcine carotid arteries for human use in the coronary or low extremity artery bypass surgeries. Major immunogenic molecules are associated with xenogenic cells, whereas major components of vascular scaffold proteins, collagens and elastins, are not immunogenic among different species because they are very conservative molecules over the evolution and had very high sequence homology among different species.
Heparin is a potent anti-coagulant that inhibits thrombin and activated factors IX, X, XI and XII, which are involved in the conversion of prothrombin to thrombin, thereby reducing thrombin formation. Our previous study has shown that heparin-coated ePTFE grafts result in reduced platelet deposition compared with non-coated control grafts in a canine femoral artery bypass model [18]. In the current study, we tested the hypothesis that the covalent linkage of the decellularized porcine arteries with heparin would provide a non-thrombogenic surface for the graft, which would reduce platelet deposition in the xenograft because thrombosis and graft patency were still major problems in vascular graft applications [6, 7, 19–21]. Heparin contents in treated vessels after culture did not show a significant reduction in both PBS and 70% alcohol. All heparin-bound vessels did not form clot during 1-hr observation, whereas the non-heparin-bound vessels were clotted in fresh dog blood within 10 min. In the baboon external shunt model, the heparin-coated vessels showed a significant less platelet deposition compared with control vessels ($P < 0.05, n = 4$).

Fig. 3 Clotting time of heparin-linked porcine arteries in dog blood. A functional clotting time assay was performed to verify the functionality of the bound heparin in vessels. Small pieces of vessels and fresh dog blood were mixed and shacked in the test tube and graft and blood clotting was observed. Decellularized vessel pieces without heparin treatment clumped together and formed clots in whole dog blood within 10 min. Decellularized vessel pieces with heparin treatment did not clot for more than 1 hr.

Fig. 4 Platelet deposition to heparin-linked porcine arteries in baboon external shunt model. Heparin-linked vessels and control vessels (5 cm long) were mounted inside of the silicon tubing, which was attached to femoral arteriovenous conduits of male baboons for 60 min. The autologous platelets of baboons were pre-labelled with $^{111}$Indium. Platelet deposition was measured by scintillation camera imaging analysis. (A) The shunt construct and experimental set-up. (B) The heparin-coated vessels showed a significant less platelet deposition compared with control vessels ($P < 0.05, n = 4$).
growth factor, which may accelerate healing and modulate remodelling process of the graft [22–24]. We previously demonstrated that the beneficial effect of local infusion of heparin in aortoiliac bypass grafting significantly reduced anastomotic neointimal hyperplasia in an animal model [25]. Similarly, we have reported the reduction of neointimal hyperplasia following heparin-coated stent placement in baboon iliac and carotid arteries compared with non-coated control stents [26, 27]. Previous study from our laboratory has also shown that the smooth muscle cells densely populated the wall and endothelial cells lined the lumen in the decellularized-heparin-treated grafts after implanted in dogs as carotid artery bypass graft [11]. Dr. Tamura reported that endothelial cells were found at the surface of decellularized-heparinized xenograft in a dog model of transplantation as the abdominal artery [13]. However, there are no comparative data showing both the heparin-treated and non-heparin-treated grafts during a relatively longer period of implantation.

In the current study, heparin is covalently linked to the decellularized graft and serves as two major purposes. The first, it can prevent the decellularized biologic graft from early thrombosis after graft implantation in vivo. The second, heparin binding growth factors such as bFGF could be loaded on the graft through high affinity interaction with heparin, which may also attract heparin binding growth factors from the circulation after graft implantation in vivo. These growth factors could promote healing and remodelling of this biologic graft. New endothelial cells and smooth muscle cells may be produced from proliferation and migration of existing vascular cells from adjacent vessels or may be differentiated from circulating progenitor cells. Once functional endothelial layer is established on the graft, anti-thrombosis property of the graft is provided by these new endothelial cells. This is a fundamental difference of the tissue-engineered vascular graft with the synthetic graft. Theoretically, tissue-engineered vascular graft after successful healing and remodelling does not require anti-thrombosis therapy because of functional endothelial cells, whereas synthetic graft requires long-term anti-thrombosis therapy because thrombogenic property of foreign materials remains after long-term graft implantation in vivo. Thus, we speculate that heparin-linked porcine carotid grafts used in the patients may need less post-operative anti-coagulation therapy compared with other types of grafts. In the current study, however, we do not know how long heparin maintains its activity on the graft in vivo. The covalent linkage was more intense in the intima than deep inside the tissue. As shown in the Fig. 1B, the covalent linkage was more intense in the intima than that in the media of the graft. However, the difference in heparin linkage between different vascular layers may not affect the development of tissue-engineered vascular grafts because anti-coagulation property at the luminal surface of the graft is the most important to prevent graft thrombosis.

In the current study, the heparin stability of the graft was tested in non-physiological conditions. It is not clear whether the heparin stability of the graft will be maintained after graft implantation in vivo. It is possible that blood haemodynamic factors, cells, chemicals or enzymes in the blood may affect the heparin stability of the graft. Thus, it is warranted to further investigate the stability of heparin of the graft under the condition of blood perfusion or in animal models.

Anti-coagulation surface of the heparin-bound decellularized graft was tested with an in vitro blood clotting time test. In principle, platelets in the fresh blood can bind to the collagen of the decellularized graft with surface collagen-specific glycoprotein Ia/IIa receptors. Blood von Willebrand factor (vWF) can help to form links between the platelets glycoprotein Ib/IX/V and the collagen fibrils, thereby activating the platelets and causing platelet aggregation. Activated platelets release the contents of stored granules into the blood plasma including several coagulation factors. These factors can induce fibrinogen accumulation and thrombin activation, which converts fibrinogens to fibrin network, thereby forming clot on the surface of the non-heparin coated graft. If heparin is coated on the surface of decellularized graft, it effectively inhibits the activation of thrombin and blocks clot formation. However, over some time (over 1 hr), fibrinolysis pathway could be activated in this in vitro system, and the clot can be dissolved. Thus, this in vitro system of the clot time test only allows reliably testing the coagulation property of the material within 1-hr time frame. In other words, if the surface is thrombogenic, the clot will form within 1 hr; whereas if the surface is non-thrombogenic, the clot will not form within 1 hr, and extending test time over 1 hr is not likely to be able to detect any clot formation.

Anti-coagulation property of bone marrow of the graft was determined by both in vitro clotting time test and ex vivo baboon model of quantitative platelet deposition assay. However, it is still not clear whether these data are applicable to in vivo situation. Thus, further investigation of anti-coagulation property of the graft in animal models is warranted.

Many attempts have been made at developing compliant small-calibre vascular grafts with varying degrees of success. One approach has been to use artificial materials. Although synthetic vascular graft materials such as Dacron or ePTFE are suitable for large-calibre (>5 mm diameter) vascular grafts, they have very poor performance when used in small-calibre applications such as cardiac bypass and infraringuinal arterial reconstructions. Another strategy for compliant small-calibre graft design is to use biological materials. Some groups have used cross-linked arteries as a basis for vascular grafts [28–31]. However, cross-linking of tissues, while reducing immune reaction and increasing strength, has some undesirable effects. One of the main problems with chemical cross-linking is residual toxicity due to reversibility of the chemical bonds between the tissue and the cross-linking agent [32]. Additionally, cross-linking may change the native condition of collagen and elastins as well as their mechanical properties. Due to these changes, cross-linked blood vessels apparently become inhospitable to cells in growth, as evidenced by no or few cells in the inner layers of cross-linked blood vessels after
implantation [28–30]. Chemically fixed vessels tend to fail beyond 2 years due to aneurismal degradation, which may be due to the breakdown of the chemical cross-links coupled with enzymatic degradation [33, 34].

In the current study, our approach is to use non-cross-linked porcine carotid artery as the basis for vascular grafts. In order to reduce immune reaction, cells and cellular debris were completely removed from the tissue by a series of enzymatic and detergent treatments. In general, collagens and elastins are highly conserved molecules in higher species, and thus even with xenogenic implants, the immune reaction should be minimal. The vessels with chemical cross-linking also retain mechanical compliance similar to native tissue as well as high strength as demonstrated before by compliance testing, burst testing and suture pullout testing [11]. In order to reduce thrombogenicity, heparin was covalently linked to the vessels in the present study. Unlike the ionic linkage of heparin used by several other groups, the covalent linkage of heparin does not require cross-linking of protein to collagens. Instead, the heparin is linked directly to the modified carboxyl end of matrix proteins such as collagens and elastins. Ionic-linked heparin also leaches out of the graft over time whereas covalent linkage should be more stable and long lasting [30]. The content of heparin in the treated vessels with covalent heparin linkage has shown no significant reduction over 2 years of storage in this study. This may be important to retain protection against thrombosis during the long period of graft endothelialization. Further long-term studies are still needed to better characterize the anti-thrombotic property of heparin-coated decellularized vessels.

Conclusions

We have developed decellularized vascular grafts with covalent linkage of heparin and performed anti-coagulant activity assay with these novel grafts. The procedure of heparin-covalent linkage is effective and reliable, which enables us to store the heparin-coated vessel for a long term without loss of heparin content. Both the in vitro clot time test and the in vivo platelet deposition assay have shown the thrombo-resistant benefits in the heparin-coating vessels. Our results suggest that heparin-coated decellularized vascular graft may represent a promising direction for the development of successful small-calibre vascular grafts for the use of clinical bypass procedures. In future, we will evaluate the vascular healing and remodelling characteristics in a large animal model such as femoral artery bypass graft in dogs or baboons. Graft safety and long-term patency are major end-points, which could be immediately translated to the human use.

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