Characterization of Antithrombotic Activity of Lumbrokinase immobilized Polyurethane Heart Valves in Total Artificial Heart Experiment

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ABSTRACT

Lumbrokinase, potent fibrinolytic enzyme purified from earthworm, was immobilized onto the total artificial heart valves using photoreaction. This valve were implanted into the lamb for three days. After experiments, thrombus was observed in the untreated valves whereas no thrombus was observed in the lumbrokinase immobilized valves. The fibrinolytic activity and proteolytic activity of the implanted valve was examined. The fibrinolytic activity of the valve was remained after the implantation. The lumbrokinase could be a suitable fibrinolytic agents in the vascular contacting devices to reduce the thrombus.

INTRODUCTION

Thrombosis is one of the major problems in vascular contacting artificial devices, including artificial vessels, ventricular assist device and total artificial hearts. In order to reduce the formation of thrombus, many biocompatible materials were used in these artificial devices[1]. Polyurethane has been used in many devices because of its good blood and tissue compatibility. In spite of its good characteristics of the biocompatibility, polyurethane also cause the thrombus in case of the long term blood contacting experiment *in vivo*[2].

To increase the biocompatibility of the biomaterials, a number of methods have been introduced in these materials including immobilization of the bioreactive materials[3]. One of the bioreactive materials used in this strategy is the materials containing the fibrinolytic or thrombolytic activity. Heparin or urokinase has been immobilized in some groups and reported to be increased the blood compatibility of biomaterial surfaces[3].

One of the major problem of the bioreactive material is the in vivo stability and function. Lumbrokinase was selected as a coating materials. Lumbrokinase was purified from earthworm *lumbricus rubellus* in 1983 for the first time. It has the potent fibrinolytic activity as well as the stability of the broad range of pH and high temperature. Due to a stability and potent fibrinolytic activity, lumbrokinase is the promising bioreactive materials in vascular contacting devices.

In previous study in our group, lumbrokinase was immobilized by using the maleic anhydride methyl ether copolymer (MACMEC) as a linker. But, the method had a problems in application of the complex three dimensional structure. In this study, lumbrokinase was immobilized using photoreactive agents as a linker and applied to the polyurethane heart valve in total artificial heart. The valve was immobilized onto the right inlet and outlet. As a control, the left inlet and outlet was not treated with lumbrokinase. In animal experiments, it was elucidated that the lumbrokinase treated valve had a reduced level of the thrombus formation.

METHODS AND MATERIALS

Purification of lumbrokinase

Lumbrokinase was purified using the modified methods of Ryu et al. Lumbrokinase fraction was pooled after treatment of ammonium sulfate to a final concentration 60 %. After dialysis against phosphate buffered saline, the pooled

solution was loaded onto the diethylaminoethyl (DEAE-sephadex) column chromatography and para-aminobenzoic acid affinity column chromatography. After passage of the column, the fractions containing the fibrinolytic activity was pooled and confirmed by SDS-PAGE. After confirmation, the purified enzyme was used in the following experiments.

Immobilization of lumbrokinase using photoreaction

The valve was coated with photoreactive polyallyl amine (0.2 %) using air spray method. After air drying, the ultraviolet light was eluminated for 5 min at the intensity of 2.5 watt to induce the amine group. The valve was washed in phosphate buffered saline for 1 days to remove the unreacted and coated polyallylamine and dried under vacuum condition. The lumbrokinase was added to the photoreactive polyallylamine coated valve to a final concentration of 100 ug/ml. EDC was added to a final concentration and incubated for 20 hours at room temperature. The valve was washed extensively to remove the adsorbed lumbrokinase and dried under vacuum.

Total artificial heart animal experiment

The healthy lamb (50 kg) was selected for the total artificial heart implantation experiment. After implant, the TAH operated for 3 days with occasional injection of heparin. The cardiac output was kept 5 liter per minutes in that period. The heparin was used as an antithrombotic agents. It was not injected for 6 hours prior to autopsy to increase the formation of thrombus.

Measurement of the fibrinolytic activity

The preparation of the fibrin plate was follows in else where. The polymer valve was incubated onto the fibrin plates at 37 °C for 24 hours. The formation of the clear zone was observed by the photography.

Measurement of the proteolytic activity

The polymer valve was incubated in 10 mg/ml azocasein in phosphate buffered saline (pH 7.4) for 24 hours at 37 °C. After incubation, 250 ul of azocasein solution was added to the 1 ml of 5 % trichloroacetic acid solution to precipitate the undegraded azocasein. The solution was centrifuged at 12,000 g for 5 minutes to separate the precipitate. The supernatant was collected and optical density of supernatant was measured at 340 nm.

RESULTS

Immobilization of the purified lumbrokinase onto the polyurethane valve

The polymer valve used in TAH was immobilized with phtoreaction. The schematic process of the reaction is shown in Figure 1.

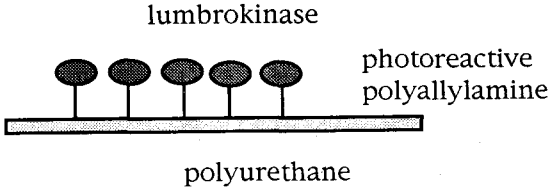


Figure 1. Schematic diagram of immobilization of lumbrokinase onto the polyurethane valve using photoreaction

The activity loss by the EDC reaction was determined to be 30 % compared with the activity of untreated lumbrokinase .

Total artificial heart experiment

The total artificial heart with the type KOTAH was consist of two blood sacs. Each contains the blood inlet and outlet.
The healthy lamb (50 kg) was selected for implantation of the TAH. The lumbrokinase treated polymer valves was assembled to the right inlet and outlet sites. After implantation, the TAH was maintained the cardiac output to 5 liter/min. It functioned for 3 days without failure. From six hours before autopsy, the antithrombotic agent (heparin) was not injected to the animal to examine the effects of the lumbrokinase. After autopsy, the valve was washed with phosphate buffered saline and cut to 4 parts for further analysis.

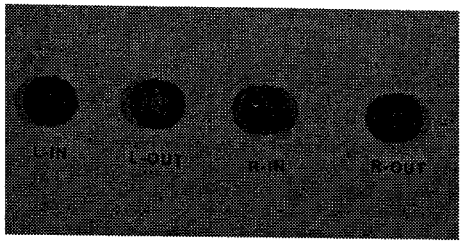


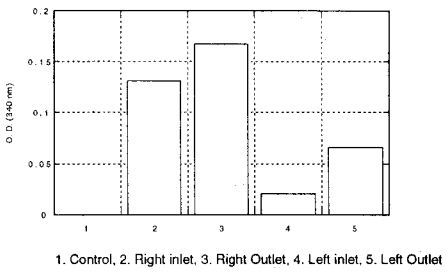
Figure 2. Photography of the heart valves after the TAH experiments

The thrombus was observed only in the left inlet valve. The size of the thrombus is 10 mm in diameter and 1-2 mm in height. No thrombi was observed in the other part of the valve; left outlet, right outlet and right inlet. The major problem of the thrombus has occurred in the inlet valve because of the low hemodynamic forces. No thrombi was observed in both part of the outlet valve whether the lumbrokinase was treated or not. But the thrombi was not observed in the lumbrokinase treated right valve whereas the big thrombi was definitely formed in the right, untreated part of the valve.

Analysis of the polymer valve assembled in TAH

The fibrinolytic activity was measured by fibrin plate methods. to verify the difference between the right and left inlet was due to fibrinolytic activity of the immobilized lumbrokinase. After incubation of the valve segment onto the fibrin plate at 37 °C for 24 hours, the clear zone was observed. That means that the immobilized lumbrokinase activity was still exist after the animal experiment.

Figure 3. Fibrinolytic activity of the LK-immobilized valve



1. Control, 2. Right inlet, 3. Right Outlet, 4. Left inlet, 5. Left Outlet

Figure 4. Proteolytic activity of the LK-immobilized valves using azocasein

Proteolytic activity

The proteolytic activity of the valve was measure by using azocasein to determine the residual activity more quantitatively. The valve segment was incubated for 24 hours in azocasein solution (10 mg/ml) and measured at 340 nm. The proteolytic activity of the right part valve was 3 times higher than that of left, thrombus formed valve (Figure 4). The residual activity of the valve after animal experiment was approximately 70 % compared the samples that did not used in animal experiment.

DISCUSSION

Lumbrokinase was immobilized using photoreaction. The photoreactive polyallylamine was used as a linker for immobilization of the lumbrokinase. The previous methods used our group had the limitation for application to the complex three dimensional structure, like a valve and artificial heart sac, because of the changes of the physical characteristics of the polyurethane. The photoreaction used in this experiment had no effects on the physical characteristics of the polyurethane. So, we can easily apply this methods for the

treatment of the valve and other complex structure.

The polymer heart valve immobilized with lumbrokinase was used to elucidate the antithrombotic effects in vivo. The lumbrokinase immobilized valve was assembled to the right outlet and inlet position and untreated valve was left outlet and inlet. This artificial heart was implanted into the lamb and was monitored for 3 days. In order to examine the effects of surface treatment, the injection of antithrombotic agent was ceased 6 hours prior to autopsy. After autopsy, the thrombus was observed only in the left inlet valve. The diameter of the thrombus was 10 mm and the height was 1 - 2 mm. It was assumed that the inlet heart valve could not be functioned properly due to this thrombi because of the decreased flexibility of the valve. This could be the cause of heart failure because the amount of the blood inlet decreased and outlet volume would be decreased and finally the blood was not supply the oxygen and other nutrients to the tissues and organs[4].

There was no thrombus in the untreated right valve. No thrombus was also found in the outlet valves. Conventionally, the formation of thrombus was not the problem in the outlet valve because of the high pressure of the blood flow. The difference of the formation of thrombus could reflect the difference of the hemodynamic difference between right and left inlet[5,6]. But, this possibility could be excluded by considering the design of the KOTAH because the structure of the KOTAH had the same in right and left sides.

The difference of thrombus formation would be the results of the difference of the surface characteristics between the treated and untreated surface, not the result of lumbrokinase activity. The lumbrokinase treated surface is more hydrophilic than native polyurethane. This would be the results of the difference because the thrombus was readily formed in hydrophobic than hydrophilic surfaces in general. Fibrin plate assay and proteolytic activity data showed that the activity of the lumbrokinase remained to a higher level after the in vivo experiment. This data showed the possibility that the fibrinolytic activity of the immobilized lumbrokinase decreased the formation of thrombus by degrading the fibrin and other coagulation related factors.

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