Biocompatibility of LK-immobilized polyurethane

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INTRODUCTION

Polyurethane (PU) is widely used as to biomaterial in blood-contacting device due to its excellent physical properties and relatively good blood compatibility with various surface modifications [1]. Lumbrokinase(LK) is a potent fibrinolytic enzyme purified from the earthworm, *Lumbricus rubellus*. In the previous study, GH Ryu. et al reported that LK-immobilized PU surface was highly antithrom-bogenic [2,3].

In this study, we investigated the effect of LK on the cell growth and the biocompatibility of the LK-immobilized PU by performing its toxity test and haemolysis test.

MATERIALS AND METHODS

Lumbrokinase purification

Lumbrokinase (LK) was purified from the earthworm (*L. rubellus*) powder using a modified method which was developed by Mihara et al. [4]. LK fractions were obtained by ammonium sulfate precipitation and diethylaminoethyl (DEAE) anion exchange column chromatography. The protein concentration of purified LK was determined by lowry protein assay. LK activity was estimated by the fibrin plate method and expressed in international units (IU) similar to the case of the urokinase.

Lumbrokinase immobilization

Two methods were used for the LK immobilization on the Polyurethane (PU) surface. The methanol-extracted PU sheets (1 x 2 cm, pellethane®2363-80AE ; Dow Chemical Co.) were immersed in 3 %(wt/vol) maleic anhydride methylvinyl ether copolymer (MAMEC) solution in tetrahydrofuran(THF). The PU sheets were coated with MAMEC solution repeatedly until uniform coating was ensured, producing the MAMEC-coated PU surface(PU-MAMEC). The other PU sheets were reacted with 1.6-Diisocyanatohexane(HMDI) in dry toluene for 1 hour at 40 °C and reacted with maleic acetic acid methylvinyl ether copolymer (MAcMEC) as linker in dry formamide. The PU sheets by two different method reacted in LK solution of 1 mg/ml in 0.05 M KH_2PO_4 (PH 4.5) for 20 h at room temperature.

LK-immobilized on PU surface was measured by the modified Bradford assay (dye-binding method). The activity of LK bounded on PU surface was measured by the caseinolytic activity assay.

Five different PU surfaces tested in this study were listed in Table 1.

PU
PU-MAMEC
PU-MAMEC-LK
PU-HMDI-MAcMEC
PU-HMDI-MAcMEC-LK

Table. 1 Five samples for biocompatibility evaluation

All samples were sterilized by the conventional ethylene oxide method.

LK effect on the cell growth

Human umbilical vein endothlial cells (HUVECs) were grown to confluence in flasks using M199 medium containing 20 % fetal bovine serum. HUVECs were harvested after 2-3 passage by

trypsinization. The cells were suspended in medium at a concentration of 10^5 cells/ml and 100 μl of cell suspension was added to 96-well tissue culture plates. Twenty-four hours after plating, the medium was removed and replaced with the defined culture medium containing different concentrations of LK solution (12.5, 25, 50, 100 nM).

The defined medium composed of M199 medium, 1 % fetal bovine serum, 1 μg/ml hydrocortisone, 50 μg/ml bovine pituitary extract, 10 ng/ml EGF, 2mM glutamine, 100 unit/ml penicillin/streptomycin. The defined medium containing LK was renewed 2 and 4 days later and HUVECs viability were checked by MTT assay.

In vitro Cytotoxicity test of LK-immobilized PU

The cell line of clone L929 mouse fibroblast was used for the cytotoxicity assay of LK-immobilized PU surface. The cells were maintained in DMEM containing 10% fetal bovine serum. Confluent L929 were harvested by trypsinization and suspended in medium at a concentration of 10^4 cells/ml and 200 μl of cell suspension was added to 96-well tissue culture plates. After the cells were near confluent in every well, extracts of test materials was added to the culture medium. Culture medium alone provided the control. After 24 hours the medium containing extracts were removed and the number of viable cells was counted by MTT assay.

L929 cells were prepared in growth medium and inoculated in 12-well tissue culture plates until the cells become confluent and overlaid with 3% noble agar in medium. The test material (1 x 1 cm) was placed on an agarose surface directly overlaying a growing cell monolayer and incubated for 24 hours at 37°C. Cell culture in the presence of agar without test materials was used as control. After 24 hours the cells were microscopically evaluated according to ASTM Standard F895-84 [5].

Haemolysis effect of LK-immobilized PUs

The ASTM Standard F756-87 hemolysis test protocol was used [6]. Test tubes containing the materials were incubated with fresh diluted rabbit blood for 4 hours at 37°C. The tubes were centrifuged and the optical density (OD) of the supernatant was measured at 540 nm. The positive reference(100% lysis) was a blood/water mixture and the negative reference (0% lysis) was a blood/saline mixture.

RESULTS AND DISCUSSION

Figure 1 shows that the level of O.D in MTT assay is linealy related with the number of the viable cells. Based upon this results, we could evaluated the effect of LK on the cellular growth by the level of the optical density in the MTT assay. The soluble LK shows no significant effect on the cellular growth as like Figure 2. The cytotoxic effect of the sample PUs shows also no significant effect on the EC growth. Inhibition of cell growth by the sample PUs were summarized in Figure 3. There exists no significant difference between the samples.

The level of the hemolysis was almost zero. The LK-immobilized PU have no effect on the hemolysis of the contacting blood.

Based upon the results of above exprements, we concluded that LK-immobilized PU is not toxic and could be a candidate materials for blood-contacting devices.

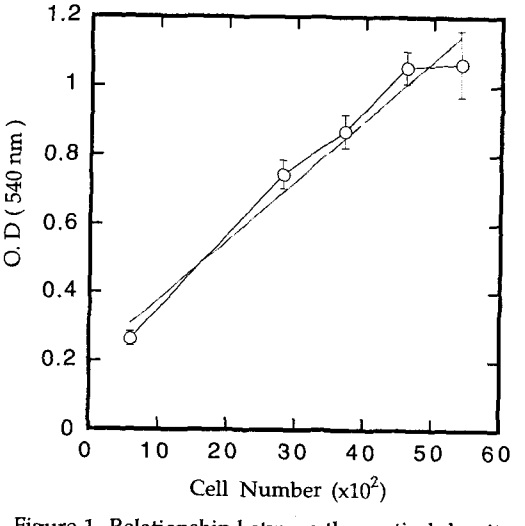

Figure 1 Relationship between the optical density level and the cell number in MTT assay

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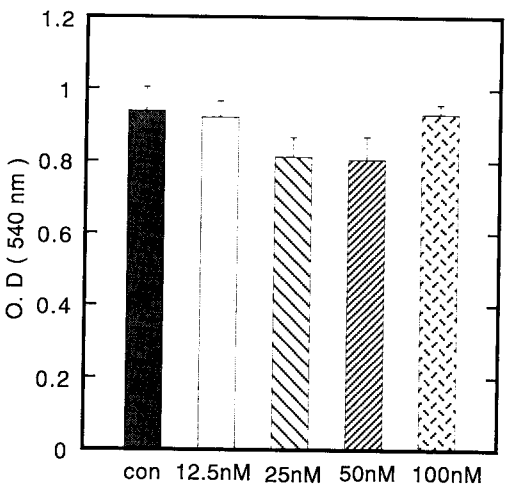

Figure 2 Endothelial cell viability checked by the MTT assay , after 3 day cell growth with the medium containing control or LK solution at different concentrations. Results are expressed as absorbance units

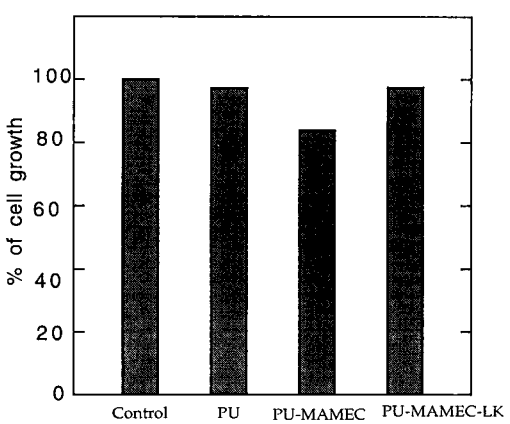

Figure 3 MTT assay of materials extracted at 121°C for 2 hours Results are expressed a percentage of the control growth(100%)