Studies on Effects of Hemodialysis Membranes on Activation of Blood Cells

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Abstract

End stage renal disease (ESRD) is the last stage of chronic kidney disease where the kidneys are functioning below 10 percent of their normal function. It requires renal replacement therapy to treat ESRD patients, such as hemodialysis (HD). HD is one of blood purification therapies, which removes waste products and excess fluid directly from the blood via HD membranes in the dialyzer. HD treatment is of critical necessity for ESRD patients. However, HD may also cause undesirable influence on ESRD patients. Negative (adverse) effects caused by HD are strongly associated with low biocompatibility of the membranes. Therefore, it is very important to improve the biocompatibility of HD membranes.

In this research, I focused on the biocompatibility of HD membranes. Biocompatibility of biomaterials can be defined as the ability of not eliciting harmful, injurious, or reactive responses on cells, tissues and other biological systems. Blood cell activation often causes adverse events, such as coagulation, inflammation and oxidative stress, and cell activation is considered to be an important determinant of biocompatibility of HD membrane. Hence, I investigated effects of the membranes on activation of blood cells in detail.

First, I showed that the degree of platelets activation was very different depending on the membranes used. Among these five tested HD membranes, NV-U, a new hydrophilic polysulfone membrane, and CX-U, a conventional polysulfone membrane, induced the lowest and highest activation of platelets, respectively. Moreover, that degree of neutrophils activation was also significantly different between CX-U and NV-U.

Next, I analyzed the molecular mechanisms of the above phenomena on CX-U and NV-U by assessing effects of integrin antagonists on the platelet and neutrophil activation. As a result, I found that plasma-derived fibrinogen was adsorbed more to CX-U than to NV-U, and that fibrinogen on membranes induced GPIIb/IIIa-mediated platelet activation and Mac-1/αvβ3-mediated neutrophil activation, depending on the amount of adsorption. These results suggested that the amount of plasma-derived fibrinogen adsorption on the membrane is a main factor to determine cell activation.

Then, I studied effects of HD membranes on the interaction among blood cells. CX-U induced the formation of platelet-neutrophil complexes, while NV-U did not induce complex formation. Moreover CX-U-induced neutrophil activation was enhanced by platelets. On the other hand, NV-U hardly affected neutrophil activation, regardless of whether platelets were present or not. These results indicate that the ability of membranes to activate blood cells is closely related to platelet-neutrophil interaction.

In conclusion, the uses of biocompatible HD membrane with lower adsorption of fibrinogen, like NV-U is expected to reduce risks of HD-associated complications and improve quality of hemodialysis treatment.

Abbreviation

APC	allophycocyanin
ß-TG	ß-thromboglobulin
CKD	chronic kidney disease
CVD	cardiovascular disease
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
ESRD	end-stage renal disease
FITC	fluorescein isothiocyanate
GP	glycoprotein
GRGDSP	N to C cyclized -(Gly-Arg-Gly-Asp-Ser-Pro)
HD	hemodialysis
ICAM	Intercellular adhesion molecule
miniMD	mini-module dialyzer
PE	phycoerythrin
PES	polyethersulfone
PF4	platelet factor 4
PRP	platelet-rich plasma
PSf	polysulfone
PSGL-1	P-slectin glycoprotein ligand-1
PVP	polyvinylpyrrolidone
ROS	reactive oxygen species
SEM	scanning electron microscope
vWF	von Willebrand factor

General introduction

Chronic kidney disease (CKD) is a type of kidney disease characterized by a gradual loss of kidney function over time (Webster et al. 2017). The most common causes of kidney disease are diabetes and high blood pressure, and so the main treatment for CKD are lifestyle modifications and medicines to manage associated medical conditions, including high blood pressure, high glucose and high cholesterol. When CKD conditions exacerbate to the most serious stage, it is called end-stage renal disease (ESRD) and the kidney is unable to filter wastes and excess fluids from the body effectively. In order to treat ESRD patients, it requires renal replacement therapy, such as hemodialysis, peritoneal dialysis and kidney transplant. Among these therapies, chronic hemodialysis is the predominant renal replacement therapy in Japan. The number of chronic dialysis patients in Japan continues to increase every year; it has reached 329,609 at the end of 2016 (Masakane 2018). According to estimates, the number of people worldwide suffering from ESRD and requiring dialysis treatment is rising at a relatively constant rate of 5-7 percent per year.

In the hemodialysis treatment, the blood is taken from the patient by the extracorporeal circuit, passed through a dialyzer to remove waste products and fluid, and returned to the patient. The dialyzer contains approximately 10000 hollow fiber membranes with pores at the nano-scale level, and uremic toxin and fluid pass out of the blood into the dialysis fluid through pores of the membranes by diffusion and filtration mechanisms. Hemodialysis thus purify blood and is regarded as a critically important therapy for ESRD patients.

In addition to the beneficial effects of blood purification, hemodialysis may affect adversely on the body. The adverse effects are closely related to blood cell activation induced by the physical interaction between the blood and the artificial surface of the hemodialysis membranes (Hakim 1993; Itoh et al. 2006; Sirolli et al. 2002; Takemoto et al. 2010). Activated cells often cause microvascular inflammation and oxidative stress by the production of pro-inflammatory cytokines, reactive oxygen species, and various cytotoxic mediators. In the chronic HD patient, this cell activation during HD is repetitive and usually occurs three times a week; even mild activation on a chronic basis may therefore affect patient's health adversely (Hakim 1993). Particularly, cell activation during hemodialysis seems to contribute to risk for atherosclerosis-related cardiovascular disease (CVD), because inflammation and oxidative stress are well known to cause endothelium dysfunction and atherosclerosis (Kaya et al. 2012; Siti et al. 2015), and CVD is highly prevalent in the dialysis population (Cozzolino et al. 2018). Based on this information, I think that it is very essential to develop hemodialysis membranes without cell activations to improve prognosis in the HD patients; however, detailed mechanisms of cell activation in hemodialysis have not been clarified yet

In this study, I focused on effects of HD membranes on blood cells. In Chapter I, I demonstrated effects of various HD membranes on activation of platelets and neutrophils. I also analyzed the mechanisms of the cell activation using integrin antagonists, and identified receptors and their ligand involved in the cell activations. In Chapter II, I discussed effects of

HD membranes on the interaction among blood cells. Because the interaction between platelets and neutrophils results in the formation of platelet-neutrophil complexes and enhancement of cell activation, I investigated effects of two HD membranes, with different abilities to activate cells, on the formation of platelet-neutrophil complexes and successive cell activation.

Chapter I:

Effects of Hemodialysis Membranes on Activation of Platelets and Neutrophils

Abstract

Activation of blood cells during hemodialysis is considered to be a significant determinant of biocompatibility of the hemodialysis membrane because it may affect patient health adversely through microvascular inflammation and oxidative stress. This study found very different cell activation among various polysulfone (PSf) hemodialysis membranes. For example CX-U, a conventional PSf membrane, induced marked adhesion of platelets to its surface and increased surface expression of activated CD11b and production of reactive oxygen species (ROS) by neutrophils. Contrarily NV-U, a hydrophilic polymer-immobilized PSf membrane, caused little platelet adhesion and slight CD11b (integrin α subunit of Mac-1) expression and ROS production by neutrophils. Analysis of the molecular mechanisms of the above phenomena on CX-U and NV-U indicated that anti-integrin GPIIb/IIIa antibody blocked platelet adhesion, and that the combination of anti-CD11b and anti-integrin αvβ3 antibodies blocked ROS production by neutrophils. Plasma-derived fibrinogen, a major ligand of GPIIb/IIIa, Mac-1 and αvβ3 on membranes, was thus analyzed and found to be more adsorbed to CX-U than to NV-U. Moreover, comparison between five PSf membranes showed that the number of adherent platelets and neutrophil ROS production increased with increasing fibrinogen adsorption. These results suggested that fibrinogen, adsorbed on membranes, induced GPIIb/IIIa-mediated platelet activation and Mac-1/αvβ3-mediated neutrophil activation, depending on the amount of adsorption.

In conclusion, the use of biocompatible membranes like NV-U, which show lower adsorption of fibrinogen, is expected to reduce hemodialysis-induced inflammation and oxidative stress, by minimizing cell activation.

Introduction

Cell activation, induced by the exposure of blood components to "non-self" hemodialysis (HD) membranes, is frequently observed during dialysis in clinical settings and activation of blood cells is considered to be a significant determinant of HD membrane biocompatibility. Studies of platelet activation and plasma concentrations of platelet factor 4 (PF4) and β-thromboglobulin (β-TG), which are released from α-granules of platelets upon acute platelet activation, reported that all are significantly greater in HD patients (Daugirdas and Bernardo 2012). In addition, platelet aggregates or platelet-leukocyte complexes were found in the peripheral circulation of patients on HD treatment (Daugirdas and Bernardo 2012) and this observation indicates that activated platelets tend to stick to themselves or leukocytes. Studies of leukocyte activation in HD patients showed that cell surface expression of CD11b on leukocytes, a marker for the activation-dependent receptor Mac-1, was increased (Sirolli et al. 2002; Yoon, Pahl, and Vaziri 2007) and that the plasma concentrations of pro-inflammatory cytokines like tumor necrosis factor α and interleukin 6, were higher in HD patients than in healthy subjects (Kakuta et al. 2016; Pertosa et al. 2000). It has also been reported that intracellular levels of reactive oxygen species (ROS) in leukocytes, and parameters of oxidative stress were increased in HD patients (Himmelfarb et al. 2002; Kaya et al. 2012; Pertosa et al. 2000).

The clinical consequences of these HD-induced cell activations have not been well elucidated; however, the increase in various inflammatory

mediators and cytotoxic materials can induce and maintain a chronic state of inflammation and oxidative stress, which may lead to HD-associated cardiovascular and other complications (Hakim 1993). Specifically, accelerated atherosclerosis and anemia are very common complications in HD patients and their pathogenesis is closely related to HD-induced oxidative stress. In particular, ROS promote conversion of macrophages to foam cells and endothelial dysfunction in atherosclerosis (Kaya et al. 2012; Recio-Mayoral et al. 2011; Ren et al. 2013) and the oxidation of lipids, proteins and nucleic acids by ROS exaggerates renal anemia by shortening the life span of red blood cells (Khalil et al. 2016). Because atherosclerosis-related cardiovascular disease (CVD) is a principal cause of morbidity and mortality in HD patients (Cai et al. 2014; Himmelfarb et al. 2002), and anemia is also a risk factor for CVD, it is extremely important to reduce such risks if we are to improve prognosis in the HD patient.

In recent years, technological advances in membrane design, chemical composition, and sterilization methods have improved biocompatibility of HD membranes. In particular, polysulfone (PSf) membranes are most commonly used in HD therapy, because of their exceptional biofunctional characteristics such as an improved biocompatibility, a sharp cut-off property for molecular weight, and good water permeability. Several different brands of PSf dialyzer are currently available commercially; however, their membranes cannot be considered equivalent due to their different manufacturing methods, e.g. differences in the contained amount of a hydrophilic polymer, polyvinylpyrrolidone (PVP), and different sterilization methods, among

others (Hayama et al. 2004). Actual activation of blood cells during HD varied among different PSf dialyzers in clinical studies (Hidaka et al. 2012; Hoenich et al. 1996; Yamaka et al. 2014). These observations imply that different PSf membranes have different effects on blood cells. However, details of these phenomena and their mechanisms have not been clarified yet because many previous *in vitro* studies that focused on biomaterial biocompatibility evaluated just platelet adhesion and the amounts of adsorbed protein (Hayama et al. 2004; Lindon et al. 1986).

The purpose of this study was to clarify the biocompatibility of PSf membranes and its mechanisms. To this end, I examined effects of five commercially available PSf type membranes including a variant of PSf, polyethersulfone (PES) on activation of platelets and neutrophils *in vitro*, and analyzed the molecular basis of the interaction between these cells and adsorbed proteins.

Materials and Methods

HD membranes

Technical data on the five HD membranes tested are summarized in Table 1. These fiber HD membranes were obtained from commercial dialyzers; APS-SA® (Asahi Kasei Medical, Tokyo, Japan), PES-SEα eco® (Nipro, Osaka, Japan), Fx-CorDiax® (Fresenius Medical Care Japan, Tokyo, Japan), CX-U® and NV-U® (Toray, Tokyo, Japan), respectively.

I measured the thickness of the swollen surface layer of the polymer as a parameter of the membrane surface structure (Table 1). For measurement of the swollen surface layer, HD membranes were cut lengthwise into half to expose their inner surfaces and then placed on a steel disk. After the membranes were immersed into pure water, they were mounted on the stage of an atomic-force microscope (AFM, Shimadzu, Kyoto, Japan) and measured immediately in contact mode, using a triangular silicon nitride cantilever (type NP-S, Digital Instruments, Santa Barbara, CA, USA). The force-distance curve was obtained by measuring the probe-sample interaction force along with the vertical displacement of the cantilever during the process of approaching the sample (Fig. 1). The probe-sample force was estimated from the deflection of the cantilever according to Hooke's law (Cappella and Dietler 1999). The length of the curved portion in the force–distance curve was defined as the thickness of the swollen surface layer because the swollen layer on membrane surfaces causes this curvature.

Surface element composition on the inner surface of the HD

membranes was measured by X-ray photoelectron spectroscopy (ESCALAB220iXL; Thermo VG Scientific, East Grinstead, UK) equipped with a monochromatized Al Kα source (1486.6 eV) of 0.15 mm diameter operating at a voltage of 10.0 kV, a current of 5 mA and a photoelectron escape angle of 90°.

Reagents

Anti-GPIa (CD29) antibody (Clone:4B4) was purchased from Beckman Coulter (Brea, CA, USA). Anti-GPIb (CD42b) antibody (Clone: 303908), PE-conjugated anti-activated CD11b antibody (clone: CBRM1/5), PE-conjugated anti-CD11b antibody (clone: ICRF44), its isotype control, PE-conjugated mouse IgG1 (clone: MOCP-21), anti-αvβ3 antibody (clone: 23C6) and anti-CD62P antibody (clone: AK4) were purchased from BioLegend (San Diego, CA, USA). Anti-CD11b antibody (clone: CBRM1/5) was purchased from eBioscience (San Diego, CA, USA). An isotype control, mouse IgG1 (clone: MPOC-21) for anti-αvβ3 antibody and anti-CD11b antibody was purchased from BioXCell (West Lebanon, NH, USA). Cyclo-GRGDSP (GRGDSP, N to C cyclized, Cyclo-(Gly-Arg-Gly-Asp-Ser-Pro)) was purchased from AnaSpec (Fremont, CA, USA). I purchased 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of mini-module dialyzer

I prepared a mini-module dialyzer (miniMD) containing 50 hollow fiber membranes (Fig. 2). The 50 fibers obtained from the dialyzers were

placed in an open-ended polycarbonate housing 12 cm long and 5.0 mm in inner diameter. Next, both ends of the bundle were potted with liquid urethane and cured to make solid urethane. The excess solid urethane ends were then cut to make flat surfaces and open the hollow fiber ends. Finally, inlet and outlet ports were attached to the housing. The assembled miniMD had two compartments; the blood-side compartment (inside the hollow fibers) and dialysate-side compartment (outside the hollow fibers).

Preparation of whole blood, platelets and neutrophils

Venous whole blood, anticoagulated with acid citrate dextrose solution (3.8 mM citric acid, 7.5 mM trisodium citrate, 13.6 mM dextrose) or heparin (50 U/mL) was obtained from healthy blood donors and used for whole blood experiments or the preparation of cells within 30 minutes from blood collection. Data obtained with whole blood/cells from one blood donor were considered as one experiment. Heparinized human blood used for the preparation of neutrophils was mixed with an equal volume of 2% dextran in saline, and most of the erythrocytes were allowed to sediment out for 40 minutes. The leukocyte-rich supernatant was then subjected to Ficoll-Paque PLUS (GE Healthcare UK Ltd., United Kingdom) density gradient centrifugation at 1300 g for 20 minutes. Residual erythrocytes were removed by hypotonic lysis (BD biosciences, Franklin Lakes, NJ, USA) with a resulting purity of >95% neutrophils as assessed by flow cytometric analysis of the expression of CD33 and CD11b (Fig. 7B).

Adhesion of platelets to the inner surface of dialysis membrane

The HD membrane was cut lengthwise into half to expose its inner surface, placed on the plastic sheet, and incubated with whole blood for one hour at 37°C with continuous agitation. In the study of the molecular mechanisms, blood was pretreated with 2 mM GRGDSP, 10 μg/mL anti-GPIIb/IIIa antibody, 10 μg/mL anti-GPIa antibody or 10 μg/mL anti-GPIb antibody for 10 minutes at 37°C and thereafter the blood was exposed to the inner surface of the membranes in the presence of the antagonists. After incubation, the membranes were rinsed with saline three times to remove non-adherent cells. The membranes were then fixed in 2.5% glutaraldehyde in saline for one hour, rinsed several times with distilled water, and dehydrated in a graded ethanol series. The samples were critical point dried and platinum sputtered. Then the specimens were examined with a Field Emission Scanning Electron Microscope (SEM) (S-800 or S-4800, Hitachi High-Technologies Corp., Tokyo, Japan) at an accelerating voltage of 3 to 5 kV. The adherent platelets on the inner surface of HD membranes in the SEM pictures of 20 random fields (enlargement ×1500, area 4.3×10^3 μm^2) were counted manually for each specimen. When more than 100 adherent platelets were observed in one field, the count of adherent platelets was censored at 100. For evaluation of morphological change in the adherent platelets on HD membranes, photographs of 9 to 10 random fields (enlargement ×5000, area 6.1×10^2 μm^2) for CX-U, APS-SA, PES-SEα eco and Fx-CorDiax were taken. Because only a small number of platelets were observed on NV-U, photographs of five fields including adherent platelets for NV-U were taken. The shape of adherent platelets in the photographs was

categorized into five morphological forms defined as follows (Goodman 1999): round or discoid (Round), dendritic (Dendritic) or early pseudopodial, spread dendritic (Spread dendritic) or intermediate pseudopodial, spreading (Spreading), and fully spread (Fully spread).

Treatment of platelets and neutrophils with small pieces of HD membrane

The HD membranes were cut into small pieces 1-2 mm in length, rinsed with sterile saline, and stored in sterile saline until use. The small pieces of membrane (derived from 100-cm length of hollow fibers) were added to 2×10^6 neutrophils suspended in 200 μL of human plasma and incubated at 37°C for 30 minutes followed by flow cytometric analysis of intracellular production of reactive oxygen species (ROS) and cell-surface activated CD11b, as described later . In the study of the molecular mechanisms, neutrophils were pretreated with 5 mM GRGDSP, 20 μg/mL anti-CD11b antibody, 20 μg/mL anti-αvβ3 antibody, combination of 20 μg/mL anti-CD11b antibody and 20 μg/mL anti-αvβ3 antibody, or 40 μg/mL isotype control IgG (mouse IgG1κ) for 10 minutes at 37°C before adding small pieces of membrane.

Hemoperfusion of mini-MD in vitro

Fig. 2 shows the *in vitro* hemoperfusion model system. The blood-side compartment of the miniMD was connected to a peristaltic pump through silicon tube lines (TIGERS POLYMER, Osaka, Japan, 120 cm long with inner diameter 1 mm and outer diameter 3 mm) and the dialysate side compartment of the miniMD was closed to avoid any ultrafiltration. Distilled water was circulated to the blood side at 1 mL/minute for more than

5 minutes, followed by the circulation of phosphate buffered saline (PBS) for 30 minutes to clean it. Subsequently, whole blood (5 mL) was circulated at a flow rate of 1 mL/min through the circuit at 37 °C, and blood sample were collected to analyze activation of neutrophils and granulocytes, or to measure adsorbed fibrinogen. A similar silicon-tube circuit without the miniMD was used for sham perfusion.

Flow cytometric analysis of intracellular production of ROS and cell-surface activated CD11b

Whole blood was circulated through the miniMD containing HD membranes for 10 minutes, as described above. The blood was stained with PE-conjugated, anti-activated CD11b antibody and FITC-conjugated anti-CD33 antibody for the analysis of cell surface expression of activated CD11b on neutrophils. For analysis of ROS production in granulocytes, the blood was stained with DCFH-DA (0.1 mM at a final concentration) for 15 minutes at room temperature. The blood was then fixed and lysed by addition of FACS lysing solution (BD Bioscience). After several washes, the cells were suspended in PBS containing 2% fetal calf serum and 0.02% NaN_3, and subjected to flow cytometry analysis.

Isolated neutrophils treated with small pieces of HD membrane in plasma were stained with PE-conjugated anti-activated CD11b antibody and DCFH-DA (0.1 mM at a final concentration) at 37°C for 15 minutes. The neutrophils were fixed with 1 mL of 1% paraformaldehyde in PBS at room temperature for 20 minutes. After washing and resuspension in modified

Tyrode buffer (137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 0.35% BSA, 10 mM Hepes, 5.5 mM D-glucose, pH7.4), the fixed neutrophils were subjected to flow cytometry analysis.

The fluorescence intensity of the cells (5000 events) was analyzed using a Gallios flow cytometer (Beckman Coulter) and data was analyzed using FlowJo version X software (FlowJo LLC, Ashland, OR, USA). Neutrophils in whole blood were identified as CD33 low-positive cells in a CD33-FITC and side-scatter dot plot (Fig. 6A). Granulocytes in whole blood and isolated neutrophils were gated according to their size and granularity on a forward-scatter versus side-scatter dot plot (Fig. 6D and 7A). The ROS production was detected by measuring the intracellular oxidized form of DCFH on flow cytometric analysis. Results of activated CD11b expression were expressed as the mean fluorescence intensity, which was calculated as the mean fluorescence intensity of cells stained by each specific antibody subjected by the mean fluorescence intensity of cells by respective isotype-matched control antibody. Results of ROS production were expressed as the mean fluorescence intensity, which was calculated as the mean fluorescence intensity of cells stained by DCFH-DA subjected by the mean fluorescence intensity of unstained cells.

Adsorption of fibrinogen onto the HD membrane

HD membranes were obtained from the miniMD after the hemoperfusion for one hour described above. The membranes (derived from 24-cm long hollow fibers) were cut into pieces about 1 mm in length, and

washed with PBS more than three times, followed by three washes with PBS containing 0.1% skim milk and 0.05% Tween. Horse radish peroxidase-conjugated anti-fibrinogen polyclonal antibody (Abcam, Cambridge, United Kingdom) was added to the membrane and incubated for 2 hours at room temperature. After four washes with PBS containing 0.1% skim milk and 0.05% Tween, the membranes were incubated with 3,3',5,5'-tetramethylbenzidine solution (Promega, Madison, WI, USA) and the absorbance at 450 nm was then determined as an index of the adsorption of fibrinogen, using a microtiter plate reader (TOSOH, Tokyo, Japan). Relative adsorption of fibrinogen onto each HD membrane (%) was calculated by defining the adsorption of fibrinogen onto NV-U as 100%.

Statistical analysis

Data were presented as the mean ± standard error of the mean (S.E.M.). Statistical significance was determined using the SAS System (SAS Institute, Cary, NC). Statistical analysis was performed using an *F* test followed by a Student's *t*-test or Welch's *t*-test for two groups, or using Bartlett's test followed by Dunnett's, non-parametric Dunnett's, or non-parametric Tukey's multiple comparison for more than three groups. Differences were considered significant when the *P* value was <0.05.

Ethics statement

This study was reviewed by the Human Tissue Samples Ethics Committee for R&D Toray Industries, Inc. and approved by the chairperson of said committee in compliance with the Human Tissue Samples Ethics

Rules for R&D Toray Industries, Inc.　All blood donors were informed about the study procedure and provided informed consent.

Results

Number and morphological change of adherent platelets

The degree of platelet activation induced by various HD membranes was evaluated *in vitro* by analyzing the number and the morphological changes of adherent platelets on the HD membranes, with the use of whole blood.

Fig. 3 shows the number of adherent platelets on the inner surface of HD membranes. The greatest number of platelets adhered to CX-U, and many platelets also adhered to PES-SEαeco and FX-CorDiax membranes, respectively. In contrast, the number of adherent platelets was least with NV-U and relatively few platelets adhered to APS-SA.

Morphological changes in platelets adhering to membranes were assessed by categorizing their shape into five morphological forms which reflect the extent of platelet spreading (Goodman 1999) (Fig. 4). As a result, platelets on CX-U, PES-SEαeco and FX-CorDiax were mostly fully spread, spreading, or spread dendritic forms. NV-U induced minimal shape changes with mainly a round form and a few fully spread, spreading, or having spread dendritic forms. Platelets on APS-SA were spread to a greater extent than those on NV-U, with most adopting a spread dendritic shape.

These results showed that the activation of adherent platelets was very different depending on the membranes used. Among the five tested HD membranes, NV-U and CX-U induced the lowest and highest activation of platelets, respectively.

Involvement of integrin receptors in platelets adhesion

I assessed the effects of platelet adhesion receptors antagonists on platelet adhesion in order to elucidate the molecules involved in the interaction between platelets and membranes. In this study, CX-U and NV-U were used as representative membranes because CX-U induced the greatest platelet adhesion among the five tested membranes, while NV-U showed least platelet adhesion. I found that adhesion of platelets to CX-U was almost completely inhibited by an Arg-Gly-Asp (RGD)-dependent integrin antagonist, GRGDSP peptide or anti-integrin glycoprotein (GP) IIb/IIIa (GPIIb/IIIa) antibody, and inhibited to a very slight extent by anti-integrin GPIa antibody or anti-integrin GPIb antibody (Figs. 5A and 5B). Similarly, adhesion of a small number of platelets to NV-U was suppressed by GRGDSP peptide or anti-GPIIb/IIIa antibody (Figs. 5C and 5D). These results suggest that integrin GPIIb/IIIa-mediated interaction with CX-U or NV-U plays a major role in this platelet activation.

Activated CD11b expression and ROS production by neutrophils

In vitro biocompatibility of biomaterials is usually evaluated by determining platelet adhesion and the amount of adsorbed protein (Hayama et al. 2004, 2004; Lindon et al. 1986); however, leukocyte activation is also an essential issue in HD therapy. Among leukocytes, I focused on granulocytes, particularly neutrophils that are by far the most prevalent type of granulocytes in the present study because an activated neutrophil is an important source of ROS. I then measured cell surface expression of

activated CD11b and ROS production of granulocytes or neutrophils as indexes of the degree of neutrophil activation induced by CX-U and NV-U.

First, I used a whole blood sample in order to study neutrophils under physiological conditions. CX-U induced an increase in cell-surface expression of activated CD11b on neutrophils and ROS production by granulocytes in whole blood (Figs. 6C and 6F). On the other hand, NV-U caused only slight or almost no increase in cell surface expression of activated CD11b and slight production of ROS (Figs. 6C and 6F). These results suggest that activation of neutrophils was significantly different between CX-U and NV-U.

Next, I proceeded to investigate mechanisms of neutrophil activation using purified neutrophils, because whole blood is too complicated a system in which to conduct further studies. Previous studies (Bonomini et al. 1997; Itoh et al. 2008, 2006) reported that HD membranes stimulate neutrophils indirectly via CD62P-mediated platelet stimulation. However I studied whether HD membranes stimulate neutrophils directly or not—showing that CX-U increased surface expression of activated CD11b and ROS production of neutrophils in the absence of other blood cells (Fig. 7). In contrast to CX-U, neutrophils treated with NV-U showed negligible or just a slight increase in the expression of activated CD11b and the production of ROS (Fig. 7). These results suggested that CX-U could stimulate neutrophils directly; however, NV-U could hardly stimulate them. This is the report of direct stimulation of neutrophils by HD membranes and complements previous studies (Bonomini et al. 1997; Itoh et al. 2008, 2006).

In summary, CX-U significantly activated neutrophils, while NV-U hardly activated neutrophils. In addition, neutrophils can be activated by a HD membrane through at least two interactions: CD62P-mediated interaction with platelets activated by the HD membrane (Bonomini et al. 1997; Itoh et al. 2008, 2006) as well as direct interaction with the HD membrane.

Involvement of integrin receptors in neutrophil ROS production

I investigated the molecular mechanisms of neutrophil ROS production directly induced by CX-U and NV-U. The production of ROS in neutrophils directly induced by CX-U was greatly inhibited by GRGDSP peptide. Anti-CD11b (integrin α subunit of Mac-1) antibody or anti-integrin αvβ3 (αvβ3) antibody alone partially inhibited ROS production in neutrophils by CX-U; however, combining the antibodies cause additive inhibition (Fig. 8A). Similarly, the small amount of production of ROS in neutrophils induced by NV-U was inhibited by GRGDSP peptide or the combination of antibodies (Fig. 8B). These results suggest that the Mac-1 and αvβ3-mediated interaction with CX-U or NV-U plays a major role in neutrophil activation.

Adsorption of fibrinogen onto membranes

The above studies using antagonists have showed that GPIIb/IIIa, Mac-1 and αvβ3 are functionally essential in the interaction of blood cells with CX-U or NV-U; I next measured the amount of their common ligand, plasma-derived fibrinogen containing an RGD sequence, adsorbed onto HD

membranes (Fig. 9). The amount of fibrinogen adsorbed on CX-U was greater than any other membrane, whereas minimal adsorption of fibrinogen appeared on NV-U. Comparing the amount of adsorbed fibrinogen and the number of adherent platelets among the five PSf membranes, the number of adherent platelets increased with the amount of adsorbed fibrinogen (Fig. 9A). ROS production by neutrophils also tended to increase, depending on the amount of adsorbed fibrinogen (Fig. 9B). In APS-SA alone, ROS production was relatively high although adsorption of fibrinogen was not high; therefore it seemed that other factors as well as fibrinogen contributed ROS production on APS-SA.

From these results, the adsorption of fibrinogen on the membrane was closely associated with activation of platelets and neutrophils.

Discussion

In this study, I examined the effect of five commercially available PSf membranes on platelets *in vitro* and found that these membranes caused different platelet activation, as assessed by the number of and morphological changes in adherent platelets. Specifically, NV-U and CX-U respectively induced the lowest and highest activation of platelets among the tested membranes. This result is probably relevant to observations from a clinical study in which dialysis using NV-U showed smaller changes in platelet parameters than dialysis using CX-U (Yamaka et al. 2014).

In my analysis of the molecular mechanisms, I examined the involvement of major platelet integrin receptors, GPIb, GPIa and GPIIb/IIIa in platelet activation, as assessed by adhesion (Li et al. 2010; Rivera et al. 2009). GPIb is part of the GPIb-V-IX complex that functions as a receptor for von Willebrand factor (vWF) and the binding of the GP Ib-IX-V complex to vWF promotes initial platelet adhesion at sites of vascular injury. GPIa is an integrin α2 subunit and associates with GPIIa to form GPIa/IIa (also called integrin α2β1), which is very important for platelet adhesion to collagen. GPIIb/IIIa is an integrin receptor that recognizes the RGD sequence of fibrinogen and plays a major role in platelet aggregation. At a site of vascular injury, all are essential and critically required for establishment of hemostasis, while only GPIIb/IIIa was important in platelet activation by CX-U and NV-U. These results indicate that the cell-surface receptors activating platelets are the same for CX-U and NV-U despite large differences

in platelet activation. Consequently, it is considered that the type of receptor on platelets does not determine the degree of platelet activation, but that the state of membrane ligands is important, as discussed later.

In addition to platelet activation, I report that activation of neutrophils was substantially different among different PSf membranes. In particular, neutrophils were activated to a greater degree by CX-U than by any other membrane, with minimal activation by NV-U. There are few clinical studies that focus on differences in neutrophil activation among the different PSf dialyzers. However, my *in vitro* results strongly suggested that dialyzers that use these PSf membranes may cause different activation of neutrophils in HD patients.

On analysis of the mechanisms of neutrophil activation, I investigated whether HD membranes stimulate neutrophils directly or not. Regarding this point, previous studies have already showed that HD membranes indirectly stimulate neutrophils by inducing the activation of platelets and subsequent CD62P-mediated adhesion of the activated platelets to neutrophils (Bonomini et al. 1997; Itoh et al. 2008, 2006). In this study, I found that HD membranes can stimulate neutrophils through a direct interaction with the membranes. This finding complements the previous study of HD-membrane induced neutrophil activation. Taken together, it is apparent that HD membranes can activate neutrophils by both direct and indirect interaction with the membranes. I then investigated the molecular mechanisms of this direct activation of neutrophils by CX-U and NV-U, examining involvement of the major neutrophil integrin receptors, Mac-1 and

αvβ3. Mac-1, also known as the CD11b/CD18 complex, is composed of an integrin αM subunit CD11b and an integrin ß2 subunit CD18. Mac-1 is the predominant integrin adhesion molecule mediating neutrophil migration, and several ligands have been described for Mac-1, including ICAM-1, iC3 as well as fibrinogen (Altieri et al. 1990). The αvβ3 integrin is a receptor for vitronectin and fibrinogen. It is well known that it is overexpressed in malignant tumors and endothelial cells during neovascularization, and plays important roles in malignant growth and angiogenesis. The αvβ3 is also found on neutrophils and mediates the adhesion of neutrophils to the extracellular matrix (Lindbom and Werr 2002). My studies using integrin antagonists in Fig. 8 suggested that the activation of neutrophils induced by CX-U or NV-U is mediated by interaction between Mac-1/αvβ3 on neutrophils and their ligand that contains the RGD sequence. Additionally, these results indicate that the cell-surface receptors that activate neutrophils were the same for CX-U and NV-U, despite large differences in neutrophil activation. I thus believe that the type of receptor on neutrophils does not determine the degree of neutrophil activation, but that the state of ligands on the membrane (described later) is considered to be important.

On comparison of neutrophils and platelets, they had similar tendencies in their responses to the various PSf membranes because both were activated by CX-U but almost never by NV-U. Conversely, there was a large difference between platelet adhesion and neutrophil adhesion to the membrane. That is to say, firm adhesion of activated platelets is often observed on bio-incompatible membranes, such as CX-U (Yamaka et al. 2014),

while adherent neutrophils are seldom observed on CX-U after either HD treatment (Yamaka et al. 2014) or in my *in vitro* studies. This information implied that interaction of neutrophils with membranes does not cause firm adhesion, but transient or tethering "rolling" adhesion. These facts are considered to reflect properties of physiological adhesion *in vivo*; irreversible adhesion of platelets is required in normal hemostasis or thrombosis, but transient adhesion of neutrophils occurs in the process of their extravasation—the movement of neutrophils out of the blood vessel and towards the site of inflammation (Radi et al. 2001).

As mentioned above, I used antagonists to directly demonstrate that integrin receptors, GPIIb/IIIa, Mac-1 and αvβ3 are functionally important in the interaction of platelets and neutrophils with CX-U or NV-U, and that these interactions depend on the RGD sequence. In addition, previous studies have described the relationship of fibrinogen deposition and cell adhesion on biomaterials (Safiullin et al. 2015; Sivaraman and Latour 2011). Based on these results, I focused on fibrinogen, which is a major ligand of GPIIb/IIIa, Mac-1 and αvβ3 that contains the RGD sequence. I confirmed that the amount of fibrinogen adsorbed on PSf HD membranes was closely related to the number of adherent platelets and ROS production by neutrophils. Regarding APS-SA, ROS production was relatively high although adsorption of fibrinogen was not high. Therefore, other ligands, such as complement proteins that could activate neutrophils through Mac-1 and LFA-1-mediated interaction, might contribute ROS production. Summarizing the above, I deduce that fibrinogen adsorbed to PSf HD

membranes causes GPIIb/IIIa-mediated platelet activation, and Mac-1/αvβ3-mediated neutrophil activation, depending on the amount of adsorption (Fig. 10). In addition, the amount of fibrinogen adsorbed to the membranes is considered to be the main determinant of the degree of cell activation.

It is known that the hydrophilicity of the membrane surface affects the adsorption of blood components, such as plasma-derived proteins and blood cells, and many PSf based-membranes are combined with a hydrophilic polymer, polyvinylpyrrolidone (PVP) to avoid the adsorption of blood components. However, the efficiency of PVP in preventing protein adsorption onto PSf membrane surfaces was reported to differ, depending on the state of PVP on the inner surface of membranes—factors such as the amount of PVP, its molecular weight, and the degree of cross-linking between PSf and PVP (Hayama et al. 2004; Oshihara et al. 2016). I thus speculate that the different states of hydrophilic polymers such as PVP affected adsorption of fibrinogen to the five PSf membranes. My data in Table 1 on the thickness of the swollen layer of the membrane surface also supported a close association between fibrinogen adsorption and the state of hydrophilic polymers. The thickness of the swollen layer mainly reflects the thickness of the hydrophilic polymer layer on the surface, because hydrophilic polymers swell markedly in wet conditions. Table 1 indicates that NV-U, with its low adsorption of fibrinogen, had the thickest swollen layer. Thowever, CX-U, which has high adsorption of fibrinogen, had the thinnest swollen layer. I therefore believe that there was an inverse relationship between fibrinogen adsorption and the thickness of the swollen layer at the membrane surface.

Because NV-U is immobilized with a hydrophilic polymer which is different from PVP (Oshihara et al. 2016), this immobilized hydrophilic polymer is considered to contribute to the thickest swollen layer.

There is no difference in surface element compositions among CX-U, NV-U, APS-SA, and FX-CorDiax (Table 2). Compared to these PSf membranes, different element composition was observed in PES-SEαeco, which is considered to reflect different structure of its material (Table 1).

Considering this information together, different states of hydrophilic polymers on the membrane surface, including their different thickness may result in a different hydrophilic/hydrophobic nature of the membrane surfaces, and subsequent different adsorption of fibrinogen (Fig. 10).

The main limitation of my study is that this is only an *in vitro* study. Cell activation during hemodialysis plays an important role in microvascular inflammation and oxidative stress in HD patients. However the causes of inflammatory syndromes in HD patients are multifactorial and include the membrane biocompatibility, the dialysate quality and the patient-related factors, such as underlying diseases. Therefore my data are insufficient for a further discussion on clinical implications of cell activations and more detailed observations in HD patients would be necessary to develop this argument.

Finally, I will discuss the clinical implications of hemodialysis membrane biocompatibility. Clinical outcomes of platelet and neutrophil activation during HD have not been clearly elucidated; however, various evidences strongly suggest that cell activation will have clinical consequences

in HD patients, as follows.

First there is considerable clinical evidence for platelet activation during HD treatment; e.g. decreases in the platelet count, the formation of platelet aggregates and increases in platelet-derived mediators (Daugirdas and Bernardo 2012; Hidaka et al. 2012; Hoenich et al. 1996; Yamaka et al. 2014). In the chronic HD patient, this platelet activation during HD is repetitive and usually occurs three times a week. Even mild activation on a chronic basis may therefore initiate harmful changes in the microcirculation, which can lead to clinical consequences in HD patients. For example, thrombocytopenia is occasionally observed in HD treatment with PSf dialyzers (Daugirdas and Bernardo 2012; Post 2010), and increased consumption of activated platelets seems to contribute to development of this thrombocytopenia. Moreover, activated platelets tend to stick to themselves or leukocytes, and circulating platelet aggregates are most likely associated with cardiovascular risk factors. Inflammatory mediators released from activated platelets, such as PF4, βTG, thromboxane A2 (TXA2), adenosine diphosphate (ADP) and serotonin would also activate the coagulation cascade and microvascular inflammation by affecting other blood cells and endothelial cells (Daugirdas and Bernardo 2012; Yamaka et al. 2014).

In addition, there is a lot of clinical evidence demonstrating the presence of activated neutrophils and oxidative stress in HD patients (Himmelfarb et al. 2002; Kaya et al. 2012; Sirolli et al. 2002). Activated neutrophils are one of the main sources of ROS, and oxidative stress results in a variety of pathological disorders such as cardiovascular diseases and

chronic inflammatory diseases. Neutrophil-induced oxidative stress during HD treatment is thus most likely closely linked to HD-associated cardiovascular and other complications, specifically accelerated atherosclerosis and renal anemia. For instance, the uptake of oxidized LDL and remnant lipoproteins by macrophages and their conversion to foam cells causes the accumulation of cholesterol by foam cells in atherosclerotic lesions (Himmelfarb et al. 2002). Oxidative damage of erythrocytes membranes and perhaps consequential accelerated apoptosis may also exaggerate renal anemia in HD patients, by shortening the erythrocyte life span (Khalil et al. 2016).

As described above, cell activation is considered to increase risks for HD-associated cardiovascular and other complications by inducing microvascular inflammation and oxidative stress. Since complication-related CVD is a principal cause of morbidity and mortality in HD patients (Cai et al. 2014; Himmelfarb et al. 2002), it is very important to reduce these risks.

Consequently, the use of a biocompatible membrane that is not associated with cell activation, such as NV-U, is expected to reduce microvascular inflammation and oxidative stress in HD patients. Furthermore, these favorable effects may prevent the development of HD-associated complications, such as accelerated atherosclerosis and improve the prognosis of HD patients.

Tables & Figures

Table 1. Technical data concerning the hollow-fiber hemodialysis (HD) membranes tested and the thickness of the swollen layer of the inner surface of HD membranes. Thickness of swollen layer of HD membranes inner surface was estimated by the force-distance curve measured by atomic-force microscopy. Data of thickness of swollen layer are presented as mean ± S.E.M. of eight independent experiments. There were statistically significant differences between CX-U and NV-U ($p<0.01$), and between NV-U and PES-SEαeco ($p<0.01$). (Tukey's multiple comparison)

Table 1

Membranes	Materials of membrane	Inner diameter (μm)	Wall thickness (μm)	Sterilization	Thickness of swollen layer (nm)		
CX-U	PSf	200	40	Gamma-ray	5.3	±	0.4
NV-U	PSf	200	40	Gamma-ray	10.4	±	1.3
APS-SA	PSf	185	45	Gamma-ray	7.9	±	1.0
PES-SEαeco	PES	200	40	Gamma-ray	5.5	±	0.7
FX-CorDiax	PSf	185	35	Steam	7.0	±	1.0

PSf: polysulfone, PES: poly (ethersulfone)

Table 2. Element composition on the inner surface of HD membranes.

Table 2

Membranes	C (%)	N (%)	O (%)	S (%)
CX-U	81.9	3.3	12.1	2.7
NV-U	81.1	3.2	13.2	2.5
APS-SA	82.1	3.9	11.6	2.3
PES-SEαeco	75.2	3.8	16.1	4.9
FX-CorDiax	82.4	3.0	11.9	2.7

Figure 1. The force-distance curve measured by atomic-force microscopy.

(A) Process used to approximate atomic-force microscopy probe to sample.

(B) Schematic representation of the force–distance curve.

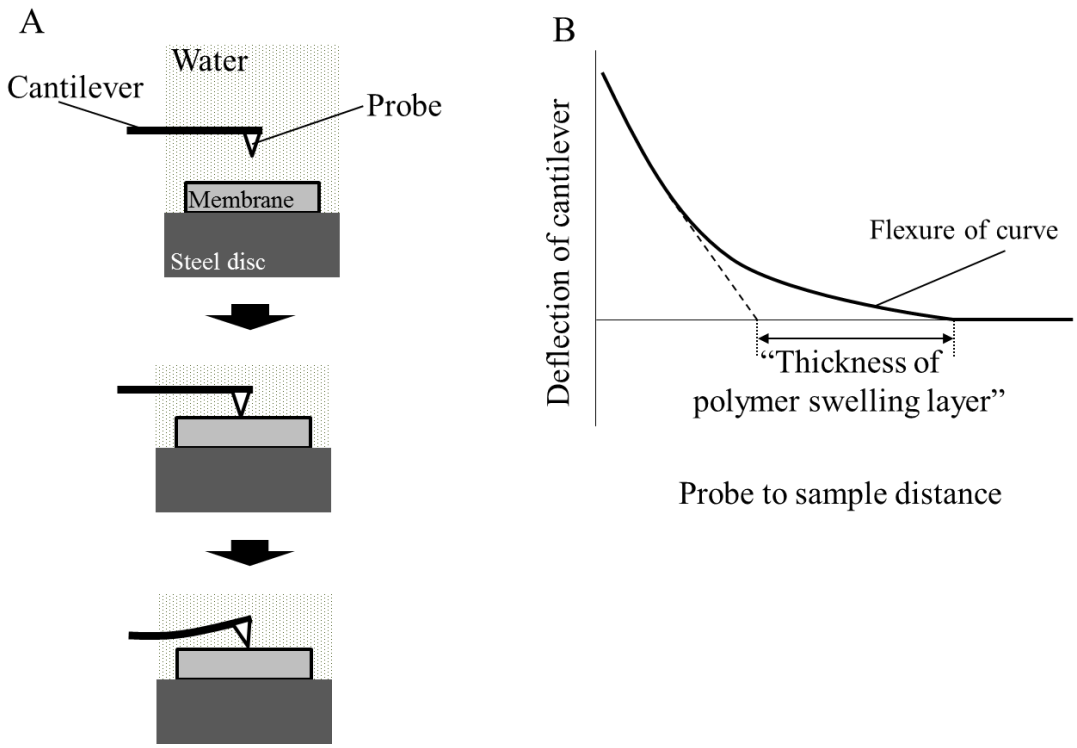

A
Water
Cantilever
Probe
Membrane
Steel disc
B
Deflection of cantilever
Flexure of curve
"Thickness of
polymer swelling layer"
Probe to sample distance

Figure 1

Figure 2. Schematic representation of mini-module dialyzer (miniMD) (A) and *in vitro* hemoperfusion model system (B).

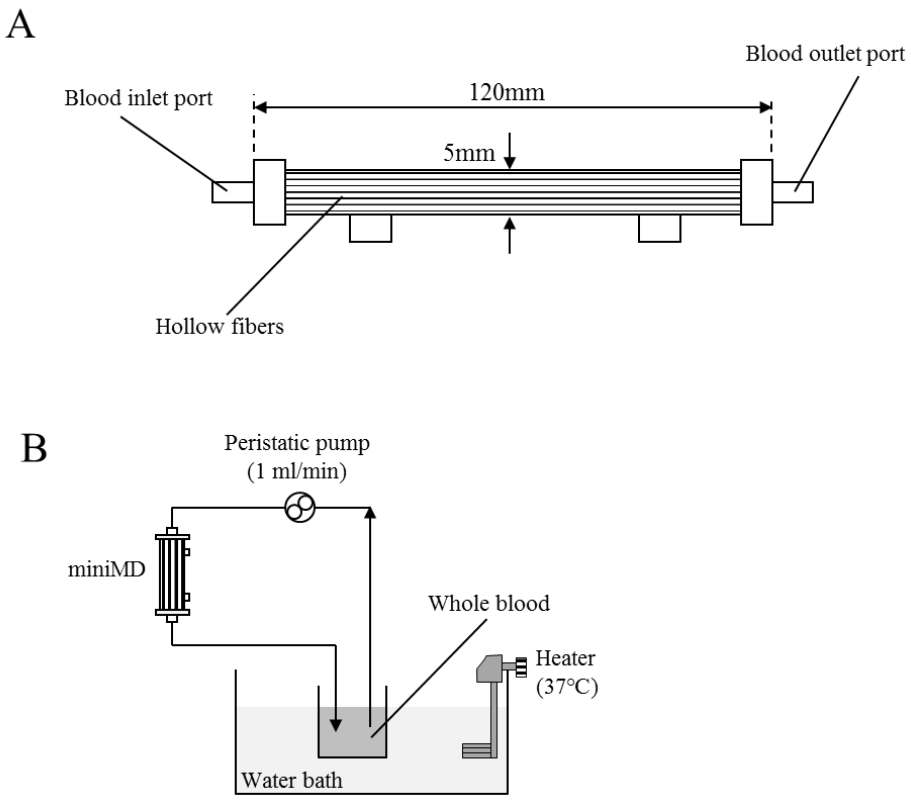

A
Blood outlet port
Blood inlet port
120mm
5mm
Hollow fibers
B
Peristatic pump
(1 ml/min)
miniMD
Whole blood
Heater
(37°C)
Water bath

Figure 2

Figure 3. Number of platelets adherent to the inner surface of hemodialysis (HD) membranes. Human whole blood was brought into contact with the inner, exposed surface of the HD membranes and incubated for one hour at 37°C with continuous agitation. The HD membranes were then washed to remove non-adherent cells and fixed with 2.5% glutaraldehyde in saline for one hour at room temperature. The inner surface of the membranes was observed with a Scanning Electron Microscope (SEM) and adherent cells were quantified by taking pictures of 20 random fields (enlargement ×1500, area 4.3×10^3 μm^2) for each specimen. The count per single field (area 4.3×10^3 μm^2) of platelets adherent to the membrane was censored at 100. Data are presented as mean ± S.E.M. of eight independent experiments (using eight different blood donors, respectively). **: $p<0.01$ between CX-U and NV-U (non-parametric Tukey's multiple comparison)

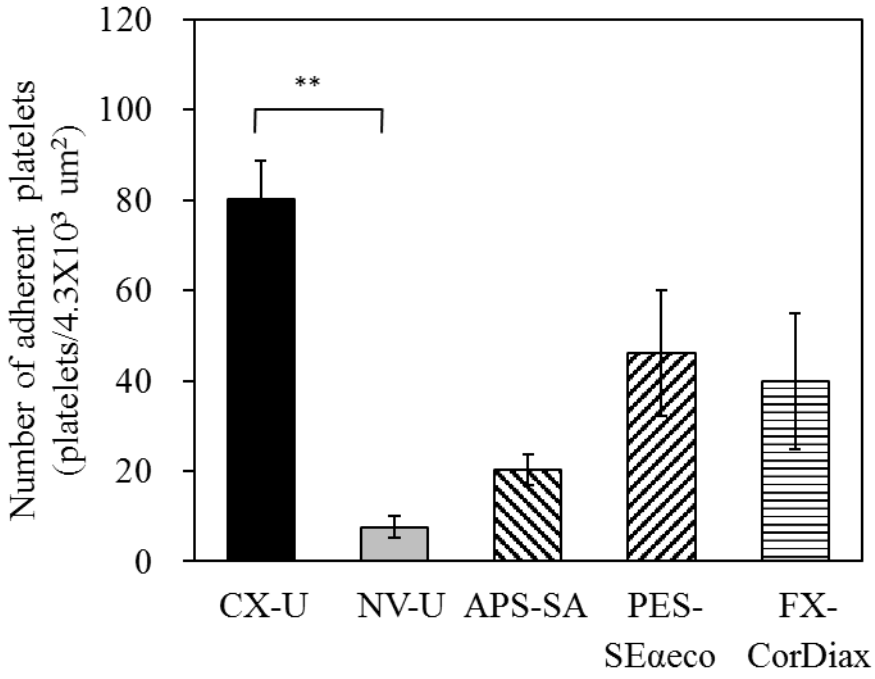

120
100
80
60
40
20
0
**
Number of adherent platelets
(platelets/4.3X10³ um²)
CX-U
NV-U
APS-SA
PES-SEαeco
FX-CorDiax

Figure 3

Figure 4. Morphological changes in platelets adherent to hemodialysis (HD) membranes. (A) Platelet shape distributions for different HD membranes. The shape of adherent platelets in the SEM pictures of five to ten fields (enlargement ×5000, area 6.1 × 10^2 μm^2) for each HD membrane was categorized. Each histogram shows the relative percentage of platelets in each of five morphological forms: round or discoid (R), dendritic (D) or early pseudopodial, spread dendritic (SD) or intermediate pseudopodial, spreading (S), and fully spread (FS). (B) Representative scanning electron micrographs of human platelets adherent to different HD membranes are shown (scale bar = 5 μm, enlargement ×10,000).

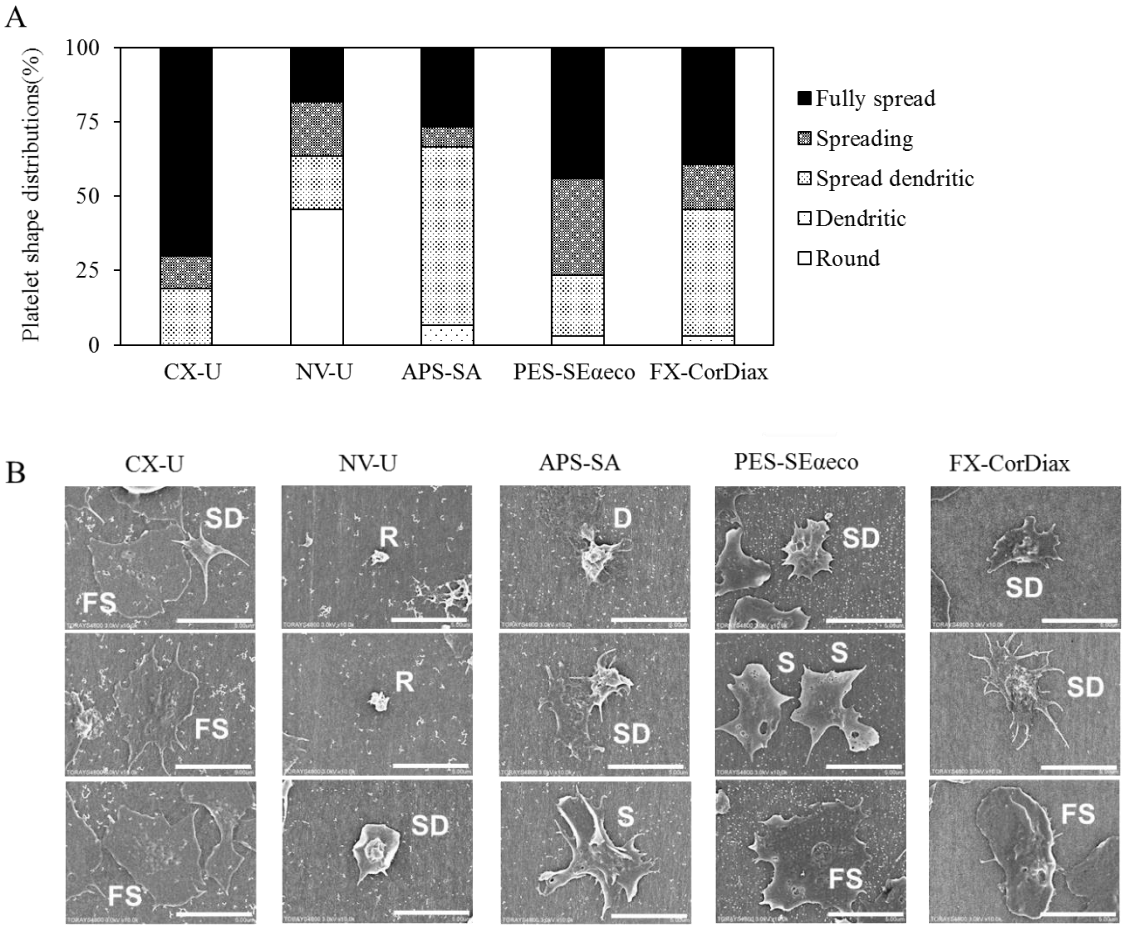

A
Platelet shape distributions(%)
100
75
50
25
0
CX-U
NV-U
APS-SA
PES-SEαeco
FX-CorDiax
Fully spread
Spreading
Spread dendritic
Dendritic
Round
B
CX-U
NV-U
APS-SA
PES-SEαeco
FX-CorDiax
SD
FS
R
D
S

Figure 4

Figure 5. Effects of integrin antagonists on adhesion of platelets to hemodialysis (HD) membranes. Whole blood was pretreated with indicated antagonists for 10 minutes at 37°C. The blood was put in contact with the inner exposed surface of the HD membranes and incubated for one hour at 37°C with continuous agitation. The HD membranes were then washed to remove non-adherent cells and fixed with 2.5% glutaraldehyde in saline for one hour at room temperature. The inner surface of the membranes was observed with a Scanning Electron Microscope (SEM) and adherent cells were quantified by taking pictures of 20 random fields (enlargement ×1500, area 4.3×10^3 μm^2) for each HD membrane. The count per single field (area 4.3×10^3 μm^2) of platelets adherent to the membrane was censored at 100. Data are presented as mean ± S.E.M of 20 SEM fields obtained from duplicate specimens from one representative experiment. The HD membranes used were CX-U (A and B) and NV-U (C and D). **: $p<0.01$ vs Control (non-parametric Dunnett's multiple comparison or Welch's *t*-test)

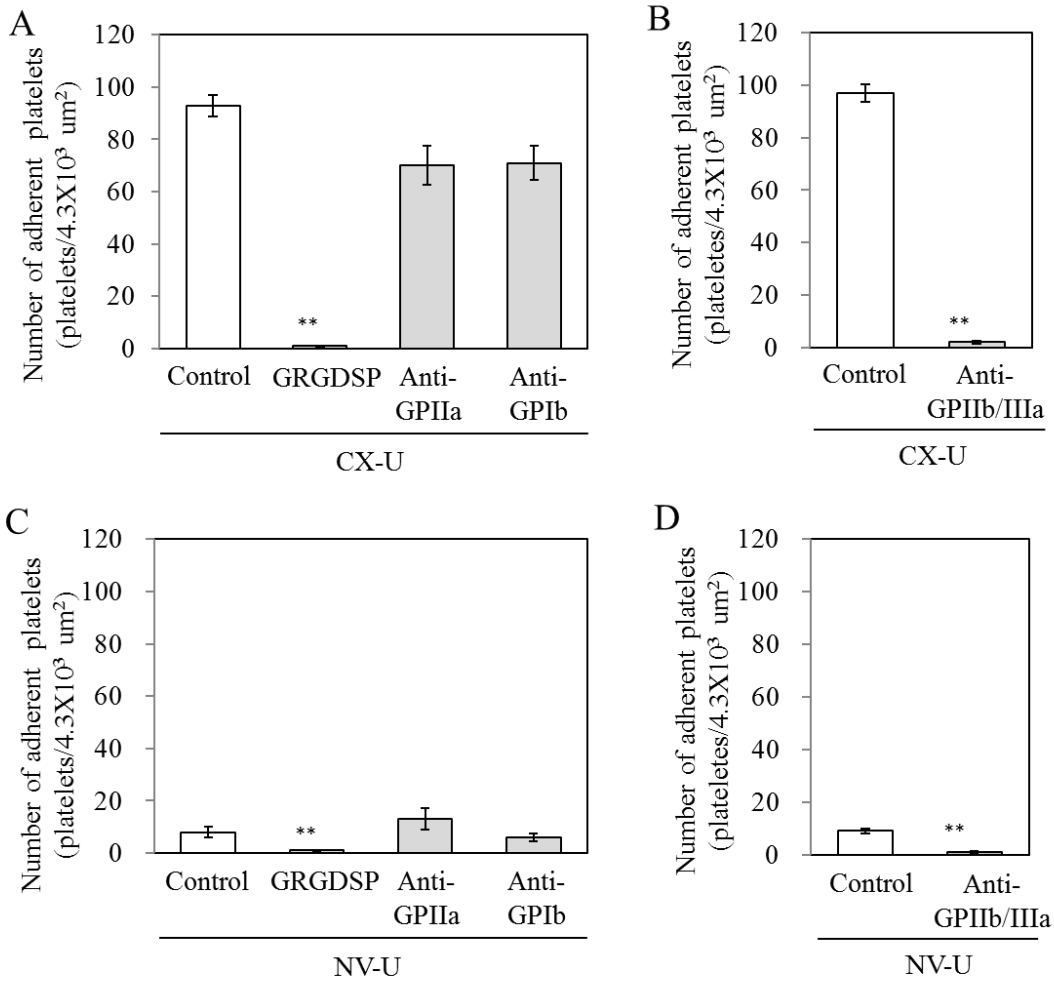

A
Number of adherent platelets (platelets/4.3X10³ um²)
120
100
80
60
40
20
0
**
Control
GRGDSP
Anti-GPIIa
Anti-GPIb
CX-U
B
Number of adherent platelets (plateletes/4.3X10³ um²)
120
100
80
60
40
20
0
**
Control
Anti-GPIIb/IIIa
CX-U
C
Number of adherent platelets (platelets/4.3X10³ um²)
120
100
80
60
40
20
0
**
Control
GRGDSP
Anti-GPIIa
Anti-GPIb
NV-U
D
Number of adherent platelets (plateletes/4.3X10³ um²)
120
100
80
60
40
20
0
**
Control
Anti-GPIIb/IIIa
NV-U

Figure 5

Figure 6. Activation of neutrophils induced by hemodialysis (HD) membranes in whole blood. Human whole blood was circulated to the mini-module dialyzer (miniMD) of CX-U or NV-U, or a silicon-tube circuit without miniMD (None group) for 10 minutes at 37°C, and thereafter stained with PE-conjugated anti-activated CD11b antibody and FITC-conjugated anti-CD33 antibody, or stained with DCFH-DA. The fluorescence intensity of cells was analyzed by flow cytometry. Neutrophils were identified as CD33 low-positive cells in forward scatter versus log fluorescence of FITC-conjugated anti-CD33 dot plot (A). (B) A representative flow cytometry histogram plot showing expression of activated CD11b on neutrophils. Filled histogram, cells with isotype control; solid line, cells without miniMD (None group); dotted line, cells of CX-U miniMD; dashed line, cells of NV-U miniMD. (C) Expression of activated CD11b on neutrophils. (D) Granulocytes were gated in forward scatter versus side scatter dot plot. (E) A representative flow cytometry histogram plot showing production of ROS. Filled histogram, autofluorescence of cells; solid line, cells without miniMD (None group); dotted line, cells of CX-U miniMD; dashed line, cells of NV-U miniMD. (F) Production of ROS on neutrophils. The ROS production in neutrophils was detected by measuring the intracellular oxidized form of DCFH. Each column represents as mean ± S.E.M. of three independent experiments (using three different blood donors, respectively) *: $p<0.05$ vs None (Dunnett's multiple comparison)

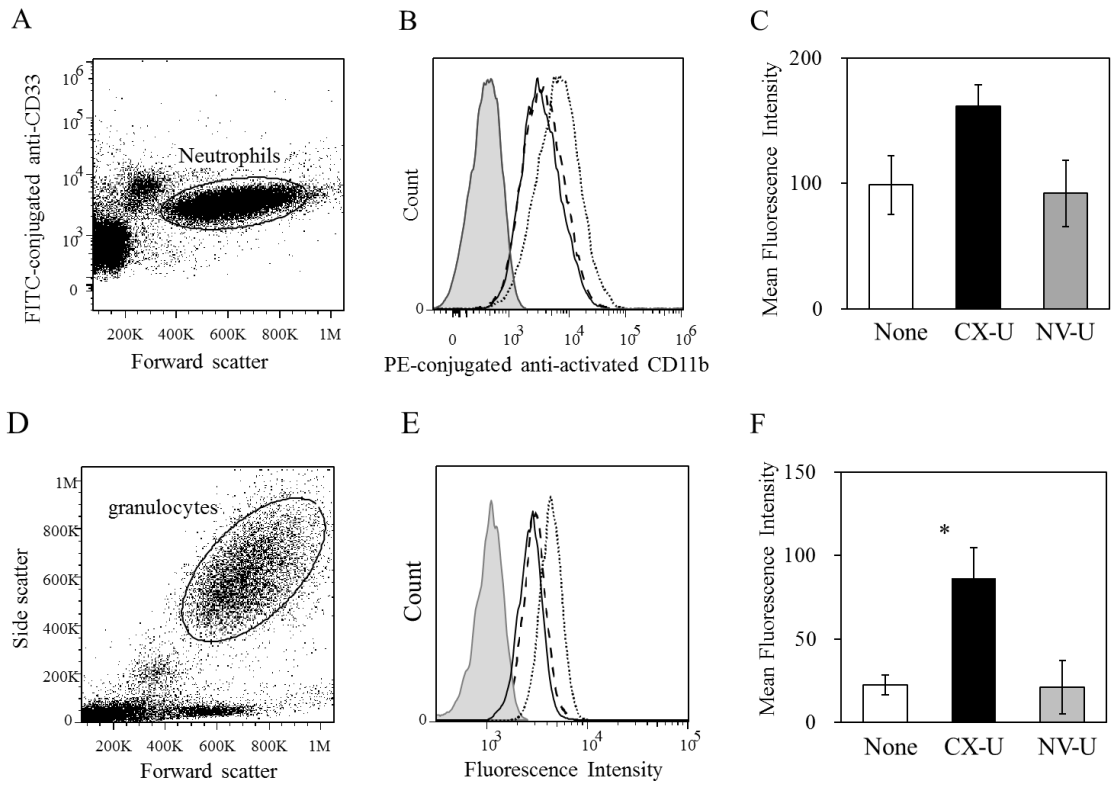

A
B
C
FITC-conjugated anti-CD33
Neutrophils
Forward scatter
Count
PE-conjugated anti-activated CD11b
Mean Fluorescence Intensity
None
CX-U
NV-U
D
E
F
granulocytes
Side scatter
Forward scatter
Count
Fluorescence Intensity
Mean Fluorescence Intensity
*
None
CX-U
NV-U

Figure 6

Figure 7. Activation of purified neutrophils induced by hemodialysis (HD) membranes. Neutrophils were isolated by dextran sedimentation followed by Ficoll-Paque density centrifugation. Isolated neutrophils suspended in plasma were treated with small pieces of HD membrane (CX-U or NV-U) or without HD membrane (None group) for 30 minutes at 37 °C, and then stained with PE-conjugated anti-activated CD11b antibody and DCFH-DA at 37°C for 15 minutes. The fluorescence of the neutrophils was measured by flow cytometry. (A) Neutrophils were gated in forward scatter versus side scatter dot plot. (B) The isolated cells were CD33 low positive and CD11b high positive in log fluorescence of FITC-CD33 versus log fluorescence of PE-conjugated anti-CD11b dot plot. The expression of activated CD11b (C) and the intracellular oxidized form of DCFH, an index of ROS production (D) were shown. Data are presented as mean ± S.E.M. of three independent experiments (using three different blood donors, respectively). *: $p<0.05$, **: $p<0.01$ vs None (Dunnett's or non-parametric Dunnett's multiple comparison)

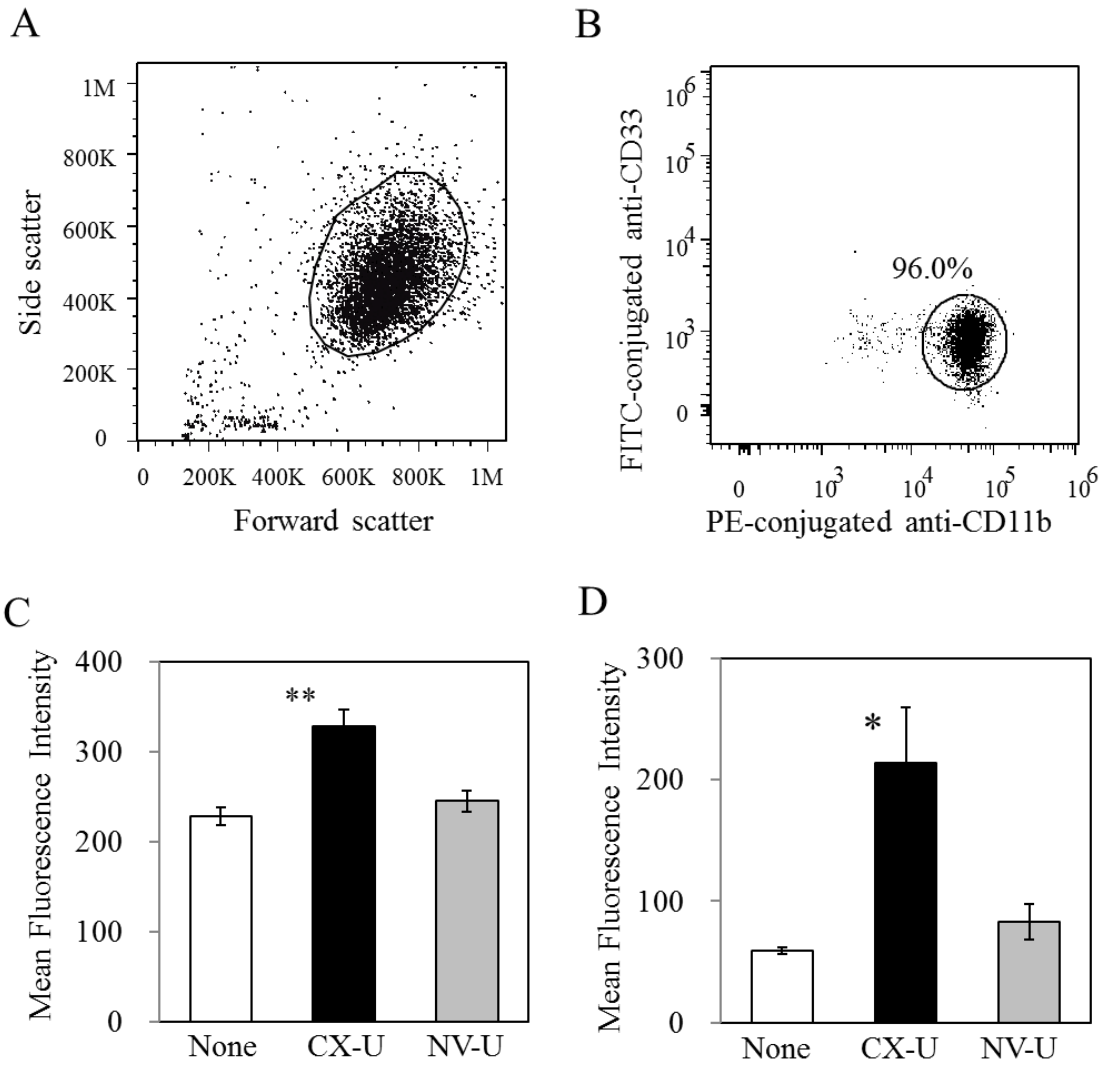

A
1M
800K
600K
400K
200K
0
Side scatter
0 200K 400K 600K 800K 1M
Forward scatter
B
FITC-conjugated anti-CD33
96.0%
PE-conjugated anti-CD11b
C
Mean Fluorescence Intensity
400
300
200
100
0
**
None
CX-U
NV-U
D
Mean Fluorescence Intensity
300
200
100
0
*
None
CX-U
NV-U

Figure 7

Figure 8. Effects of integrin antagonists on neutrophils activation induced by CX-U and NV-U. Isolated neutrophils suspended in plasma were treated with small pieces of hemodialysis (HD) membrane or without HD membrane (None group) in the absence or presence of indicated antagonists for 30 minutes at 37 °C, and then stained with DCFH-DA at 37°C for 15 minutes. The HD membranes used were CX-U (A) and NV-U (C). The fluorescence of the neutrophils was measured by flow cytometry to detect the intracellular oxidized form of DCFH, an index of ROS production. Data are presented as mean ± S.E.M. of triplicate measurements from one representative experiment. **: $p<0.01$ between Control/CX-U(-) vs Control/CX-U(+) (t-test), ##: $p<0.01$ vs Control/CX-U(+) (Dunnett's multiple comparison)

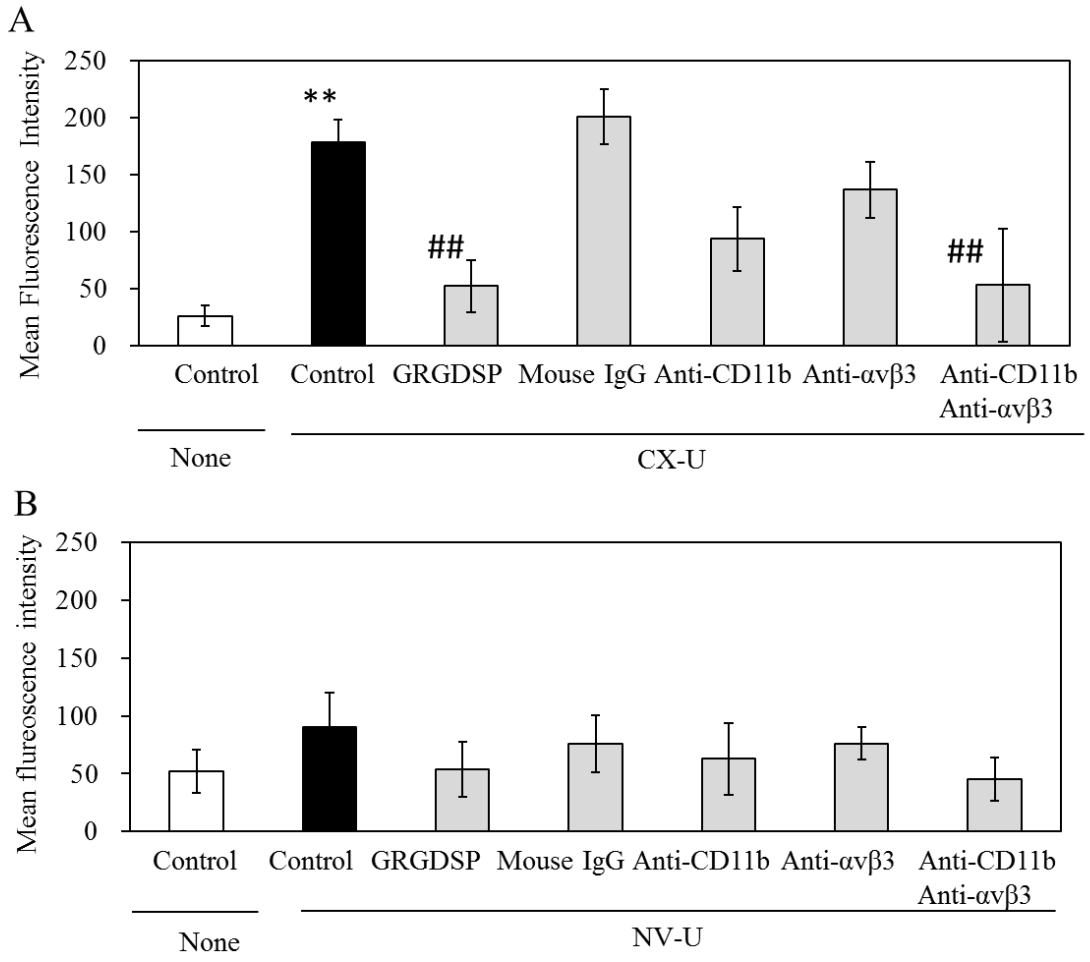
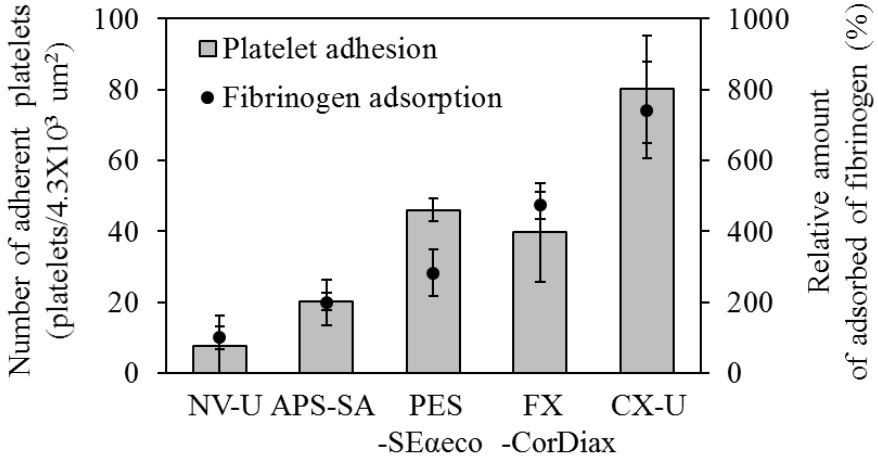

A
Mean Fluorescence Intensity
250
200
150
100
50
0
**
##
##
Control
Control
GRGDSP
Mouse IgG
Anti-CD11b
Anti-αvβ3
Anti-CD11b
Anti-αvβ3
None
CX-U
B
Mean flureoscence intensity
250
200
150
100
50
0
Control
Control
GRGDSP
Mouse IgG
Anti-CD11b
Anti-αvβ3
Anti-CD11b
Anti-αvβ3
None
NV-U

Figure 8

Figure 9. Adsorption of fibrinogen onto hemodialysis (HD) membranes and its relationship to cell responses. The right axis and black circle symbols show the relative adsorption of fibrinogen onto HD membranes determined as follows. The HD membranes were obtained from the miniMD after hemoperfusion for one hour. After the membranes were cut into pieces about 1 mm in length and washed, they were incubated with horse radish peroxidase-conjugated anti-fibrinogen antibody for 2 hours at room temperature. The membranes were then incubated with 3,3',5,5'-tetramethylbenzidine solution and the absorbance at 450 nm was determined as an index of the adsorption of fibrinogen. Data shows the relative adsorption of fibrinogen onto each HD membrane (%), defining the adsorption of fibrinogen onto NV-U as 100%. (A) The left axis and gray columns show the number of platelets adherent to each HD membrane described in Fig. 3. (B) The left axis and gray columns show the mean fluorescence intensity, which indicates ROS production of neutrophils determined as follows. Isolated neutrophils suspended in plasma were treated with small pieces of HD membrane for 30 minutes at 37 °C, and then stained with DCFH-DA at 37°C for 15 minutes. The fluorescence of the neutrophils was measured by flow cytometry to detect the intracellular oxidized form of DCFH, an index of ROS production. Data for fibrinogen adsorption and ROS production are presented as mean ± S.E.M. of three independent experiments.

A


Number of adherent platelets (platelets/4.3X10³ um²)
Relative amount of adsorbed of fibrinogen (%)
Platelet adhesion
Fibrinogen adsorption
100
80
60
40
20
0
1000
800
600
400
200
0
NV-U
APS-SA
PES -SEαeco
FX -CorDiax
CX-U

B

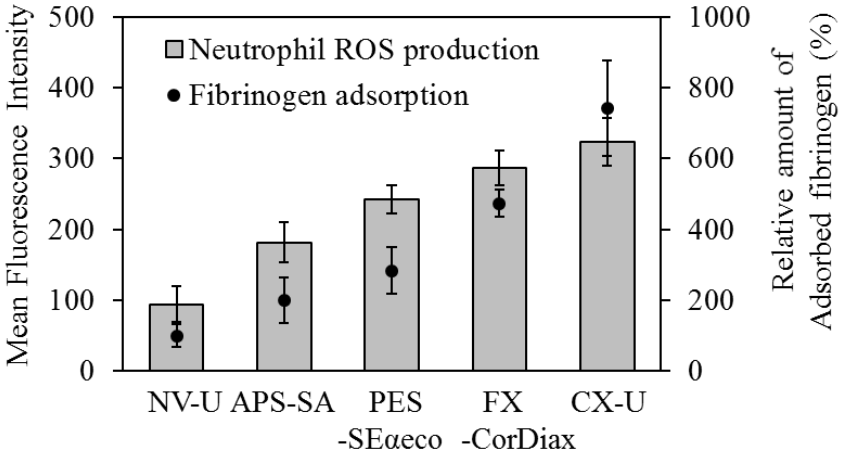

Mean Fluorescence Intensity
Relative amount of Adsorbed fibrinogen (%)
Neutrophil ROS production
Fibrinogen adsorption
500
400
300
200
100
0
1000
800
600
400
200
0
NV-U
APS-SA
PES -SEαeco
FX -CorDiax
CX-U

Figure 9

Figure 10. Schematic illustration showing the means by which hemodialysis membranes affect platelet and neutrophil activation.

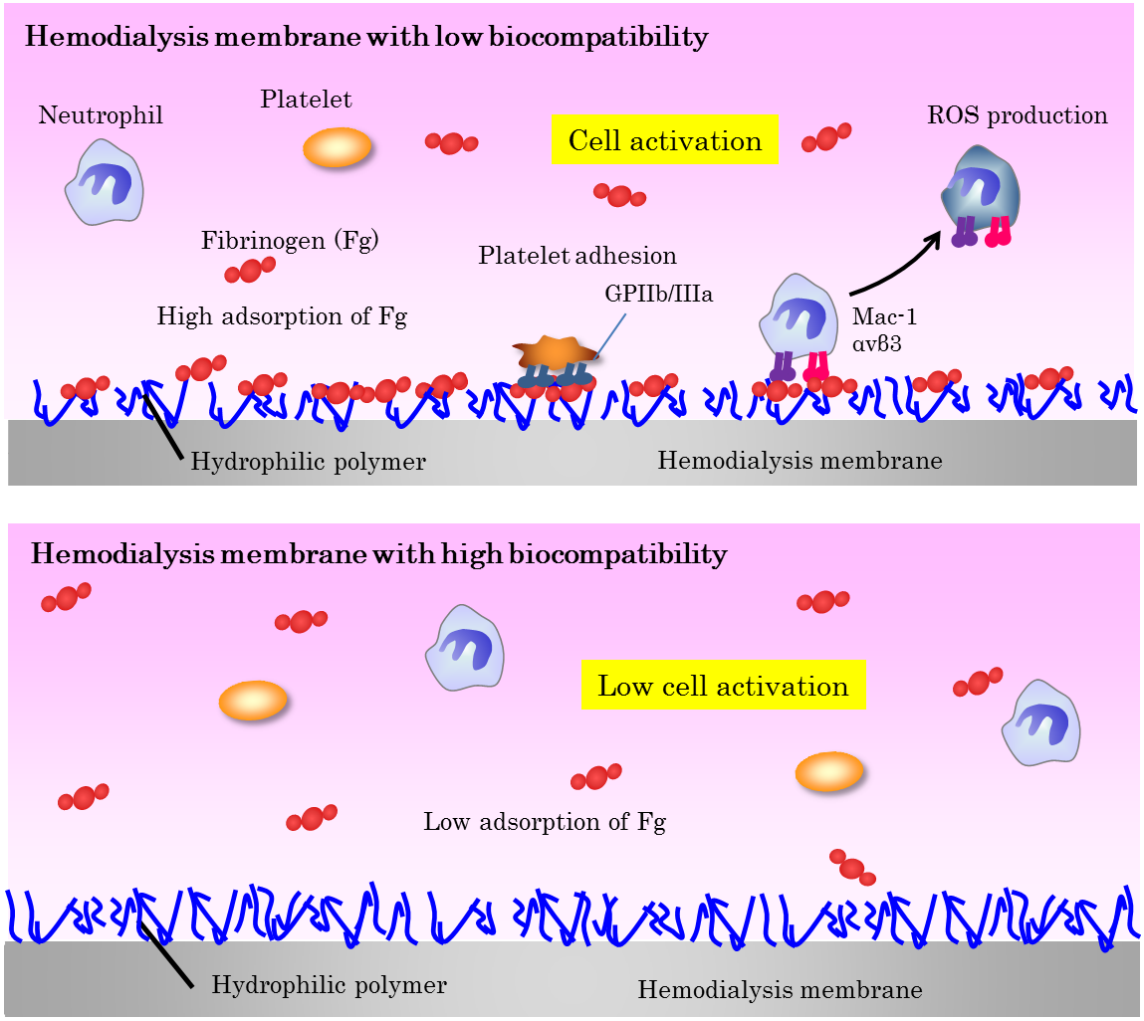

Hemodialysis membrane with low biocompatibility
Neutrophil
Platelet
Cell activation
ROS production
Fibrinogen (Fg)
Platelet adhesion
GPIIb/IIIa
High adsorption of Fg
Mac-1
αvβ3
Hydrophilic polymer
Hemodialysis membrane
Hemodialysis membrane with high biocompatibility
Low cell activation
Low adsorption of Fg
Hydrophilic polymer
Hemodialysis membrane

Figure 10

Chapter II:

Effects of Hemodialysis Membranes on Platelet–Neutrophil Interactions and Successive Neutrophil Activation

Abstract

Microaggregates have often been observed during hemodialysis and are clearly associated with complications of hemodialysis therapy. In this study, I aimed to clarify the effects of two polysulfone (PSf) membranes, with different abilities to activate blood cells, on the formation of these microaggregates. I also investigated their molecular mechanisms.

Human whole blood was circulated through a mini-module dialyzer using the membranes *in vitro*, and platelet-neutrophil complexes in blood were determined by flow cytometry. Isolated human neutrophils were incubated with the membranes in plasma in the presence or absence of platelets, followed by flow cytometric analysis of intracellular reactive oxygen species (ROS) and cell-surface activated CD11b on neutrophils.

CX-U, a conventional PSf membrane with remarkable cell activation, induced the formation of platelet-neutrophil complexes. However, NV-U, a new hydrophilic PSf membrane with slight or no cell activation, did not cause complex formation. Moreover, CX-U-induced ROS production and the increase in activated CD11b expression on neutrophils were enhanced by platelets. Conversely, NV-U hardly affected neutrophil activation, regardless of whether platelets were present or not. The enhancement of CX-U-induced neutrophil activations by platelets was greatly inhibited by anti-CD62P antibody.

The ability of PSf membranes to activate blood cells is closely related to platelet-neutrophil interaction. Therefore, a biocompatible membrane,

like NV-U, can be expected to prevent microaggregate formation during hemodialysis and avoid subsequent cell activation.

Introduction

The formation of microaggregates has often been observed in blood sample of hemodialysis (HD) patients. Various studies have shown that the process involves interaction between blood cells and HD membranes, particularly HD membrane-induced platelet activation (Bonomini et al. 1997; Gawaz et al. 1994, 1999; Sirolli et al. 2002), and the platelet-neutrophil complex is primarily mediated by the interaction between CD62P on activated platelets and its ligand, P-selectin glycoprotein ligand-1 (PSGL-1), on neutrophils (Itoh et al. 2008, 2006). In addition, several studies suggested that direct physical interaction with platelets primes neutrophils to enhance neutrophil functions, such as production of reactive oxygen species (ROS), leading to oxidative stress-related pathological conditions (Bonomini et al. 1997).

A heightened incidence of microaggregates also exists in a variety of inflammatory, auto-immune and infectious diseases such as sepsis, inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis, chronic obstructive pulmonary disease, and various bacterial infections (Gawaz et al. 1995; Johansson et al. 2011; Pamuk et al. 2006). Moreover, heightened occurrence of microaggregates has been encountered in cardiovascular disease, including atherosclerosis and ischemia-reperfusion injury (Ott et al. 1996; Setianto et al. 2010). This information suggested that microaggregates may contribute to the inflammatory and thrombotic processes in HD patients. In particular, atherosclerosis-related

cardiovascular disease is a major cause of morbidity and mortality in HD patients (Cai et al. 2014), and I believe that it is important to reduce such risks by preventing microaggregate-associated inflammation and thrombus formation.

Microaggregate formation during HD was reported to depend on the membrane materials. Regenerated cellulose (RC), and modified cellulosic membranes and polysulfone (PSf) membranes were somewhat more active than ethylene vinyl alcohol and polyacrylonitrile (Gawaz et al. 1994, 1999; Sirolli et al. 2002). Among these materials, PSf is most commonly used to constitute HD membranes because of its high performance and recent technological advances led to the development of a new class of PSf membranes. Several different brands of PSf dialyzer are currently available commercially, and their membranes cannot be considered to be equivalent because I demonstrated their different activities against cells in Chapter I. However, it is not clear whether such variability causes parallel differences in the formation of microaggregates. In this study, I examined the effects of two PSf membranes, which have different abilities to activate cells, on the formation of platelet-neutrophil complexes and subsequent neutrophil activation.

Materials and Methods

Hemodialysis membranes

The hemodialysis (HD) membranes were obtained from commercial dialyzers: TORAYLIGHT® CX-U and TORAYLIGHT® NV-U (Toray, Tokyo, Japan). The same polysulfone (PSf)-based membrane (inner diameter: 200 μm, wall thickness: 40 μm, sterilization: Gamma-ray irradiation) is used in both CX-U and NV-U, but the surface of NV-U is improved through the use of a new hydrophilic polymer (Oshihara et al. 2016).

Reagents

Anti-CD62P antibody (clone: AK4), FITC-conjugated anti-CD33 antibody (clone: HIM3-4) and its isotype control, FITC-conjugated mouse IgG1 (clone: MOPC-21), APC-conjugated anti-CD41 antibody (clone: HIP-8) and its isotype control, APC-conjugated mouse IgG1 (clone: MOPC-21), PE-conjugated anti-activated CD11b antibody (clone: CBRM1/5) and its isotype control, PE-conjugated mouse IgG1 (clone: MOPC-21) were purchased from BioLegend (San Diego, CA, USA). I purchased 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of whole blood, platelets and neutrophils

Venous whole blood anticoagulated with heparin (50 U/mL) was obtained from healthy blood donors and used within 30 minutes of blood collection. Data obtained with whole blood/cells from one blood donor were

considered as one experiment. Platelet-rich plasma (PRP) was prepared by centrifuging heparinized blood at 150 g for 10 minutes. To ensure autologous, cell-free plasma collection, heparinized blood was centrifuged at 1400 g for 10 minutes, followed by filtration with a 0.2 μm filter. Heparinized human blood used for the preparation of neutrophils was mixed with an equal volume of 2% dextran in saline, and most of the erythrocytes were allowed to sediment out for 40 minutes. The leukocyte-rich supernatant was then subjected to Ficoll-Paque PLUS (GE Healthcare UK Ltd., United Kingdom) density gradient centrifugation at 1300 g for 20 minutes. Residual erythrocytes were removed by hypotonic lysis (BD biosciences, Franklin Lakes, NJ, USA) with a resulting purity of >95% neutrophils as assessed by flow cytometric analysis of the expression of CD33 and CD11b.

This study was reviewed by the Human Tissue Samples Ethics Committee for R&D Toray Industries, Inc. and approved by the chairperson of the committee in compliance with the Human Tissue Samples Ethics Rules for R&D Toray Industries, Inc. All blood donors were informed about the study procedure and provided informed consent.

Hemoperfusion of miniMD in vitro

I prepared a mini-module dialyzer (miniMD) containing 50 hollow fiber membranes (Fig. 2 in Chapter I). The blood-side compartment of the miniMD was connected to a peristaltic pump through silicon tube lines (TIGERS POLYMER, Osaka, Japan, 120 cm long with inner diameter 1 mm

and outer diameter 3 mm) and the dialysate side compartment of the miniMD was closed to avoid any ultrafiltration. Distilled water was circulated through the miniMD at 1 mL/minute for more than 5 minutes, followed by the circulation of phosphate buffered saline (PBS) for 30 minutes to clean it. Subsequently, whole blood (5 mL) was circulated at a flow rate of 1 mL/min through the circuit at 37°C for 10 minutes, and blood samples were collected for analysis. A similar silicon-tube circuit without the miniMD was used for sham perfusion.

Treatment of platelets and neutrophils with small pieces of HD membrane

The HD membranes were cut into small pieces 1–2 mm in length, rinsed with sterile saline, and stored in sterile saline until use. The small pieces of membrane (derived from 100-cm lengths of hollow fibers) were added to 2×10^6 neutrophils suspended in 200 µL of autologous plasma or PRP and incubated at 37°C for 15 minutes, followed by flow cytometric analysis of intracellular production of reactive oxygen species (ROS) and cell-surface activated CD11b, as described later. In the study of molecular mechanisms, neutrophils were pretreated with 20 µg/mL anti-CD62P antibody for 10 minutes at 37°C before adding small pieces of membrane.

Flow cytometric analysis of platelet-neutrophil complexes, intracellular production of ROS and cell-surface activated CD11b

The blood sample collected after hemoperfusion *in vitro* was stained with FITC-conjugated anti-CD33 antibody and APC-conjugated anti-CD41 antibody. The blood was then fixed to prevent spontaneous activation of

platelets and lysed by addition of FACS lysing solution (BD Bioscience); the cells were suspended in PBS containing 2% fetal calf serum and 0.02% NaN_3, and subjected to flow cytometry analysis within one hour following sample preparation.

Isolated neutrophils treated with small pieces of HD membrane in plasma were stained with PE-conjugated anti-activated CD11b antibody and DCFH-DA (0.1 mM at a final concentration) at 37°C for 15 minutes. After diffusion into the cell, DCFH-DA is deacetylated by esterases to the non-fluorescent compound DCFH, which is later oxidized by ROS into a fluorescent compound. The neutrophils were fixed with 1% paraformaldehyde in PBS at room temperature for 15 minutes. After washing and resuspension in modified Tyrode buffer (137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 0.35% BSA, 10 mM Hepes, 5.5 mM D-glucose, pH7.4), the fixed neutrophils were subjected to flow cytometry analysis.

The fluorescence intensity of the cells (more than 5000 events) was analyzed using a Gallios flow cytometer (Beckman Coulter). CD41 positive and CD33 low-positive cells were identified as the platelet-neutrophil complexes (Figs. 11A and 11B). Results of formation of platelet-neutrophil complexes were expressed as the percentage of CD41 positive cells (%) among CD33 low-positive cells which was calculated as the percentage of cells stained by anti-CD41 antibody subjected by the percentage of cells stained by an isotype control antibody. The isolated neutrophils were gated according to their size and granularity on a forward-scatter versus side-scatter dot plot.

ROS production was detected by measuring the intracellular oxidized form of DCFH on flow cytometric analysis. Results of activated CD11b expression were expressed as the mean fluorescence intensity, which was calculated as the mean fluorescence intensity of cells stained by each specific antibody subjected by the mean fluorescence intensity of cells by respective isotype-matched control antibody. Results of ROS production were expressed as the mean fluorescence intensity, which was calculated as the mean fluorescence intensity of cells stained by DCFH-DA subjected by the mean fluorescence intensity of unstained cells.

Statistical analysis

Data were presented as the mean ± standard error of the mean (S.E.M.). Statistical significance was determined using the SAS System (SAS Institute, Cary, NC). Statistical analysis was performed using an F test followed by a Student's t-test for two groups or using Bartlett's test followed by Dunnett's comparison for three groups. Differences were considered significant when the P value was <0.05.

Results

In this study, CX-U and NV-U were used as representative membranes because CX-U induced the greatest cell activation among the five test membranes, while NV-U showed least cell activation (Fig. 9 in Chapter I). At first, I investigated the effects of CX-U and NV-U, on the formation of platelet-neutrophil complexes in whole blood. To evaluate the interaction among blood cells under flow conditions similar to those found in the clinical setting, I used an *in vitro* miniMD system that pumps whole blood through HD membranes. Circulation of whole blood through the miniMD with CX-U increased the formation of platelet-neutrophil complexes compared to sham circulation without a miniMD. On the other hand, NV-U had no effect on the formation of platelet-neutrophil complexes (Fig. 11). This result indicated that CX-U promoted the interaction between platelets and neutrophils; however NV-U didn't affect it.

I then explored the influence of platelet-neutrophil complexes on cell activation and its mechanisms. In this experiment, I used purified neutrophils and platelet rich plasma (PRP) to conduct experiments under well-defined conditions suitable for assessing the interaction between platelets and neutrophils. When neutrophils were incubated with CX-U in the absence of platelets, CX-U induced ROS production of the neutrophils and increased activated CD11b expression on the neutrophils (Figs. 12 and 13). In the presence of platelets, the CX-U-induced neutrophil ROS production and activated CD11b expression were further increased (Figs. 12 and 13). On the other hand, NV-U did not affect neutrophil activation whether

platelets were present or not (Figs. 12 and 13). These result suggested that platelets enhanced CX-U-induced neutrophil activation.

Because CD62P, also called P-selectin, is known to mediate platelet-neutrophil adhesion (Itoh et al. 2008, 2006; Zarbock et al. 2007), I then examined the involvement of CD62P in the enhancement of neutrophil activation induced by platelets. Anti-CD62P antibody reduced CX-U-induced neutrophil ROS production in the presence of platelets to the same level of production seen in the absence of platelets (Fig. 14A). In addition, anti-CD62P antibody reduced activated CD11b expression in the presence of platelets (Fig. 14B). In the presence of platelets, there were no statistically significant differences between controls and anti-CD62P in terms of ROS production (p=0.09) or activated CD11b expression (p=0.10).

Discussion

Clinical evaluation reports have shown that the number of platelets adhering to membrane surfaces was lower with the NV-U dialyzer than with CX-U (Yamaka et al. 2014). As my *in vitro* studies in Chapter I also supported these clinical observations, I was able to confirm that CX-U caused remarkable activation of platelets but NV-U did not. CX-U and NV-U have very different abilities to activate blood cells; I thus further investigated their biocompatibility by assessing their effects on the formation of microaggregates and subsequent cell activation *in vitro*.

My results can be summarized as follows: 1) CX-U increased the formation of platelet-neutrophil complexes, and platelets promoted CX-U-

induced neutrophil activation; 2) The enhancement of CX-U-induced neutrophil activation by platelets was greatly suppressed by the blockade of CD62P; 3) NV-U did not provoke the formation of platelet-neutrophil complexes and neutrophil activation, even when co-incubated with platelets. On comparing CX-U and NV-U, the cell activation induced by the membranes seems to correlate with the formation of platelet-neutrophil complexes induced by the membranes because the formation of cell complexes was induced to a great degree by CX-U, with minimal changes induced by NV-U. Regarding platelet activation, previous studies suggested that the formation of microaggregates induced by HD membranes depends on the ability of the HD membranes to activate platelets (Gawaz et al. 1994; Itoh et al. 2006). This is because CD62P is usually stored in a membrane protein granule in platelets and rapidly expressed on the cell surface to establish cell adhesion, but only when platelets are activated (Zarbock et al. 2007). In agreement with this information, it is thought that the different abilities of CX-U and NV-U to stimulate platelets led to different CD62P expression on the surfaces of platelets, followed by differential formation of platelet-neutrophil complexes.

Regarding neutrophil activation, the redistribution of a CD62P ligand, PSGL-1, on neutrophils is thought to contribute to the formation of platelet-neutrophil complexes (Itoh et al. 2008). In particular, with regenerated cellulose (RC) membranes, complement-induced redistribution of PSGL-1 on neutrophils is considered to be the main cause of microaggregate formation. This is because neutrophils constitutively express PSGL-1 and the RC

membrane showed a slight tendency towards platelet activation (Itoh et al. 2006). PSf membranes activate complement poorly (Jorstad et al. 1988) and it is quite unlikely that complement-induced redistribution of PSGL-1 occurred. My study in Chapter I showed that fibrinogen adsorbed on PSf membranes can activate neutrophils, therefore, CX-U-induced neutrophil activation via fibrinogen may cause redistribution of PSGL-1 and subsequent microaggregate formation.

Microaggregates of blood cells are often observed in a variety of pathological disorders including HD treatment, and inflammatory disease (Gawaz et al. 1995; Johansson et al. 2011; Pamuk et al. 2006). The clinical outcomes associated with increased microaggregates have not been clearly elucidated; however, they play important roles in cell activation, ROS production, cell adhesion and phagocytosis (Bonomini et al. 1997; Peters et al. 1999). In fact, I confirmed that interaction with platelets augmented CX-U-induced neutrophil ROS production. Thus, HD-membrane-induced microaggregates are most likely linked to HD-associated complications by inducing chronic inflammation and oxidative stress, and I believe that it is extremely important to reduce these risks.

The main limitation of my study is that I assessed the biocompatibility of HD membranes by assessing the formation of microaggregates *in vitro*, as it is sometimes difficult to interpret clinical data because inflammatory syndromes in HD patients are associated with a variety of factors, such as membrane biocompatibility, dialysate quality and patient-related factors, such as underlying disease. However, the

biocompatibility of HD membranes should be evaluated in the clinical setting, and more detailed observations in HD patients are thus necessary for further discussion on the clinical implications of my results.

In summary, I demonstrated that varying platelet and neutrophil activation among HD membranes causes parallel differences in the formation of microaggregates. It is very important to prevent cell activation if we are to prevent microaggregate formation. Consequently, the use of HD membranes with low inducibility for cell activation, like NV-U, is expected to decrease the formation of microaggregates in HD patients, and have some benefits in hemodialysis treatment by reducing the risk of various HD-associated complications.

Figures

Figure 11. Formation of platelet-neutrophil complexes induced by CX-U and NV-U in whole blood. (A) A dot plot of forward scatter versus log fluorescence of the FITC-conjugated anti-CD33. The CD33 low-positive cells were identified as neutrophils. (B) A representative flow cytometry histogram plot showing expression of CD41 on CD33 low-positive cells. (C) The percentage of CD41 positive cells among CD33 low-positive cells (platelet-neutrophil complexes) was determined. Data presented as mean ± S.E.M. of three independent experiments are shown. The *p* value shown was obtained by Dunnett's multiple comparison.

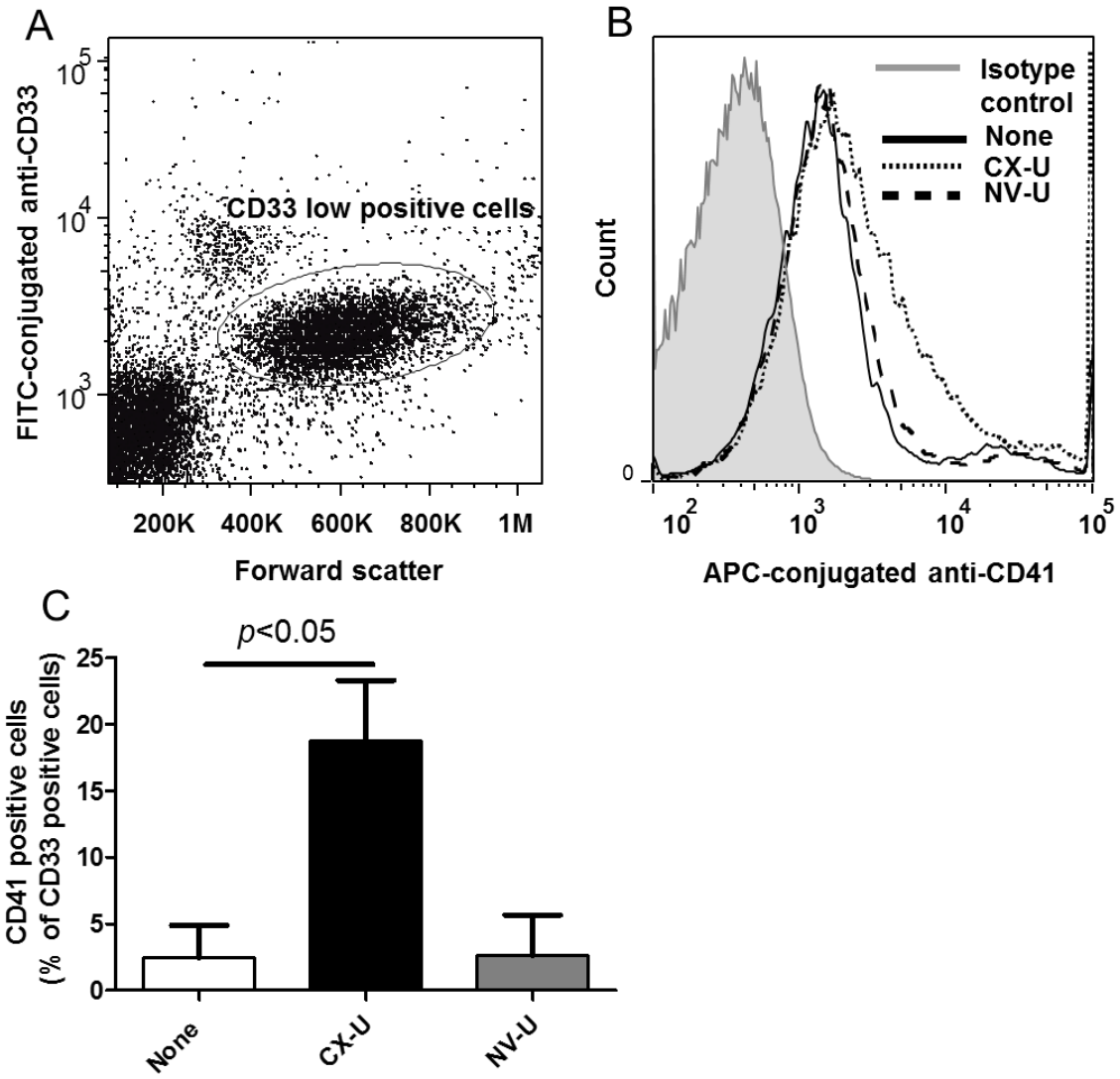

A
FITC-conjugated anti-CD33
CD33 low positive cells
Forward scatter
200K
400K
600K
800K
1M
B
Isotype control
None
CX-U
NV-U
Count
APC-conjugated anti-CD41
C
p<0.05
CD41 positive cells
(% of CD33 positive cells)
None
CX-U
NV-U

Figure 11

Figure 12. Enhancement of hemodialysis (HD) membrane-induced neutrophil ROS production by platelets. Isolated neutrophils suspended in plasma (platelet (-)) or platelet rich plasma (platelet (+)) were treated without (None) or with an HD membrane, and then stained with DCFH-DA. Representative flow cytometry histogram plots (A, B, C) and data presented as mean ± S.E.M. of four independent experiments (D) are shown. The *p* value was obtained from a *t*-test.

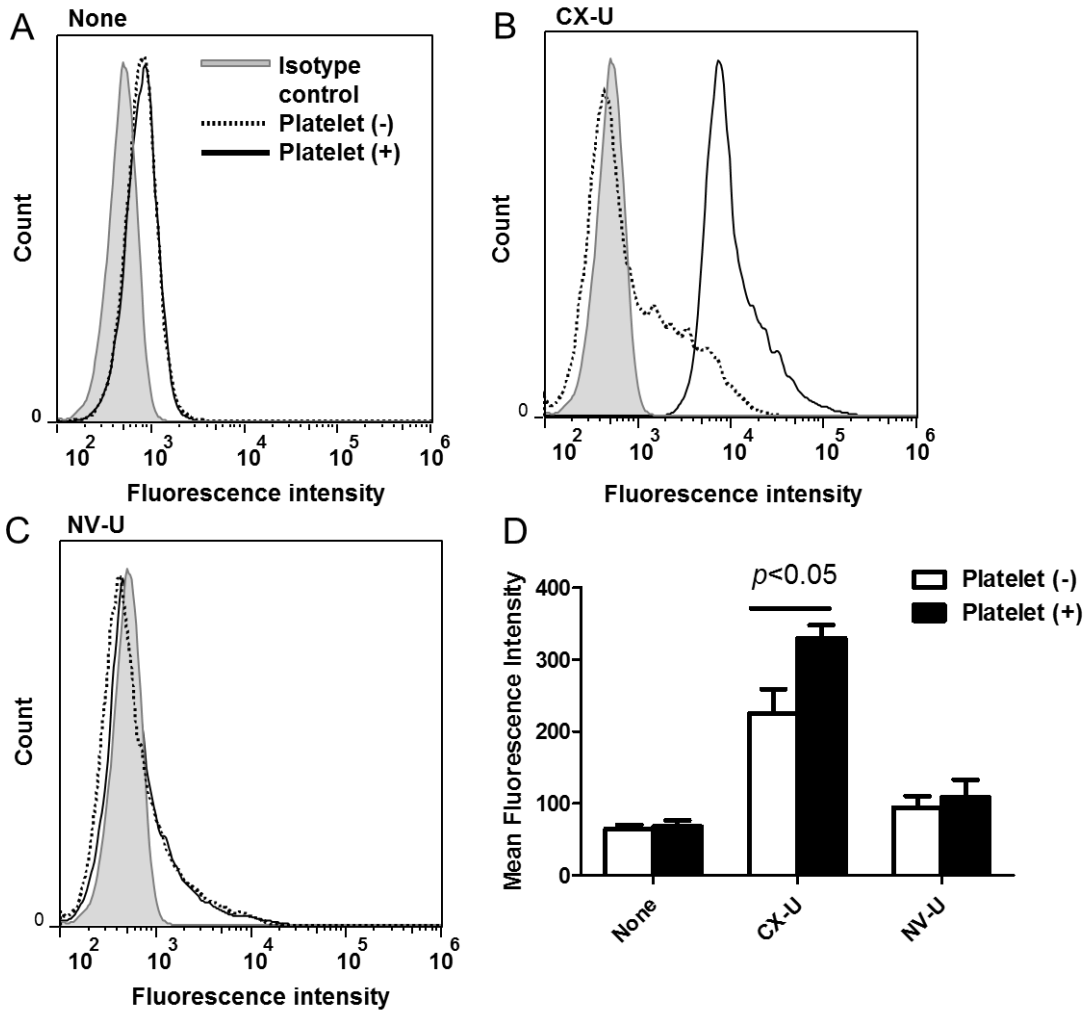

A
None
Isotype control
Platelet (-)
Platelet (+)
Count
0
10^2 10^3 10^4 10^5 10^6
Fluorescence intensity
B
CX-U
Count
0
10^2 10^3 10^4 10^5 10^6
Fluorescence intensity
C
NV-U
Count
0
10^2 10^3 10^4 10^5 10^6
Fluorescence intensity
D
p<0.05
Platelet (-)
Platelet (+)
Mean Fluorescence Intensity
400
300
200
100
0
None
CX-U
NV-U

Figure 12

Figure 13. Enhancement of activated CD11b expression of neutrophils by platelets. Isolated neutrophils suspended in plasma (platelet (-)) or platelet rich plasma (platelet (+)) were treated without (None) or with an HD membrane, and then stained with PE-anti-activated CD11b antibody. Representative flow cytometry histogram plots (A, B, C) and data presented as mean ± S.E.M. of three independent experiments (D) are shown. The *p* value was obtained from a *t*-test.

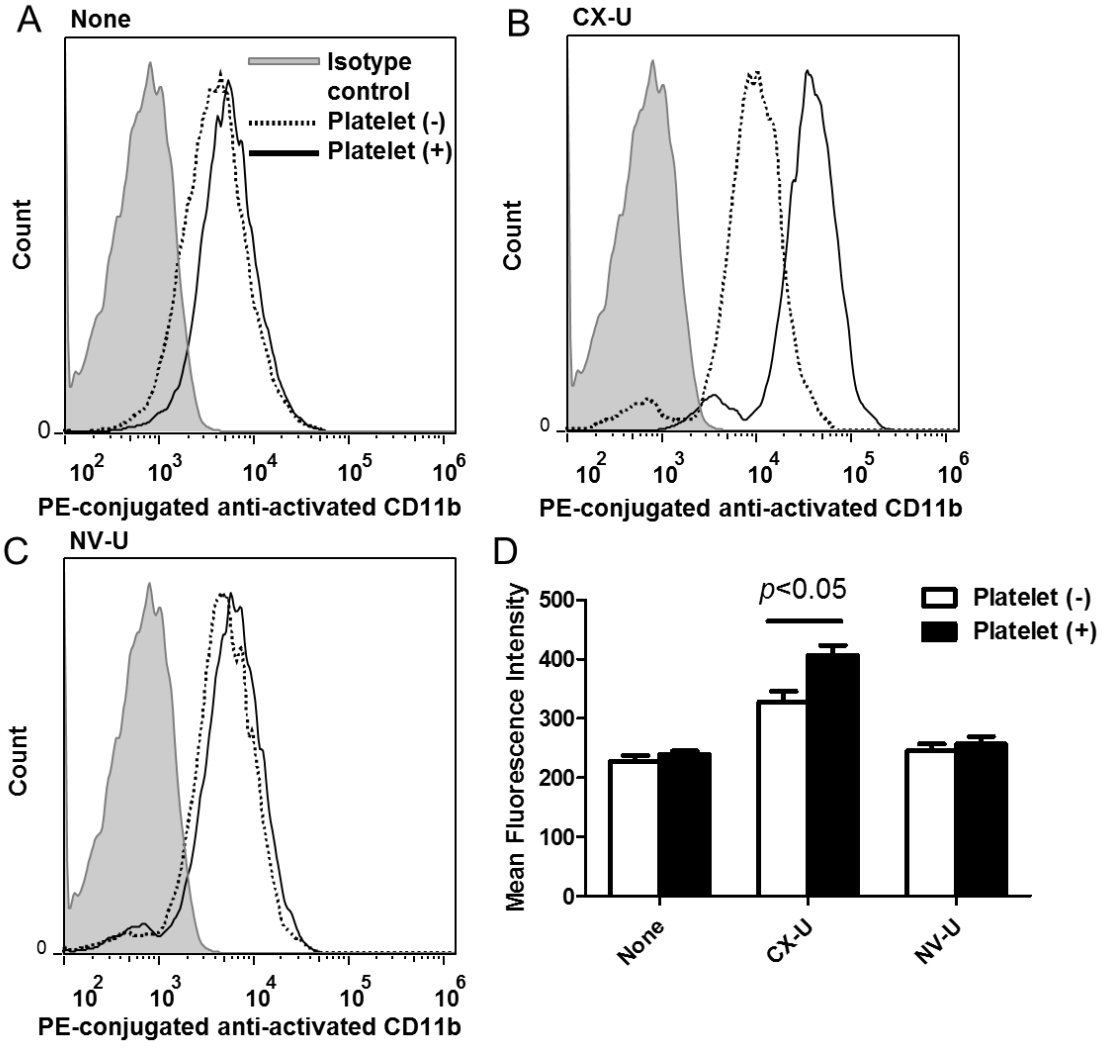

A
None
Isotype control
Platelet (-)
Platelet (+)
Count
0
10^2 10^3 10^4 10^5 10^6
PE-conjugated anti-activated CD11b
B
CX-U
Count
0
10^2 10^3 10^4 10^5 10^6
PE-conjugated anti-activated CD11b
C
NV-U
Count
0
10^2 10^3 10^4 10^5 10^6
PE-conjugated anti-activated CD11b
D
p<0.05
Platelet (-)
Platelet (+)
Mean Fluorescence Intensity
500
400
300
200
100
0
None
CX-U
NV-U

Figure 13

Figure 14. Effects of anti-CD62P antibody on hemodialysis (HD) membrane-induced neutrophil activation. Isolated neutrophils suspended in plasma (platelet (-)) or platelet rich plasma (platelet (+)) were treated with or without small pieces of CX-U in the absence or presence of 20 μg/mL anti-CD62P antibody, and then stained with PE-conjugated anti-activated CD11b and DCFH-DA. Data are presented as mean ± S.E.M. of triplicate measurements obtained from one representative experiment. The *p* values shown were obtained from *t*-tests.

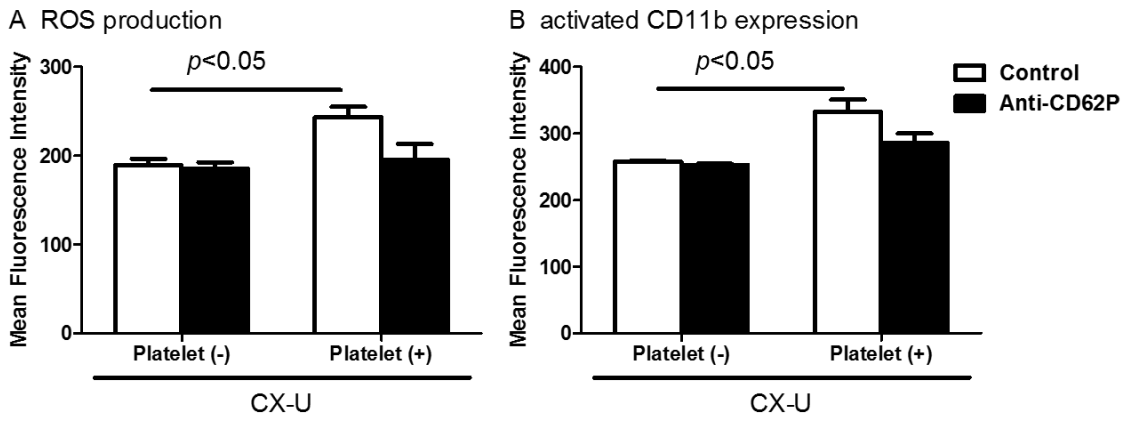

A ROS production
p<0.05
Mean Fluorescence Intensity
300
200
100
0
Platelet (-)
Platelet (+)
CX-U
B activated CD11b expression
p<0.05
Mean Fluorescence Intensity
400
300
200
100
0
Platelet (-)
Platelet (+)
CX-U
Control
Anti-CD62P

Figure 14

General discussion

In Chapter I, I assessed the effects of five PSf HD membranes on blood cells *in vitro*, and showed that the number of platelets adherent to their surfaces, and the amount of ROS produced by neutrophils were clearly different among membranes. For example, CX-U, a conventional PSf membrane, induced adhesion of many platelets and increased the surface expression of activated CD11b and ROS production of neutrophils; on the other hand, NV-U, a newly developed hydrophilic PSf membrane, had virtually no effect on platelets and neutrophils. Study of the molecular mechanisms of these cell activations demonstrated that adhesion of platelet and ROS production by neutrophils were mediated by GPIIb/IIIa on platelets, and Mac-1 and αvβ3 on neutrophils, respectively. In addition, the number of adherent platelets and the amount of neutrophil ROS production increased with the increase of the amount of fibrinogen adsorbed on the membranes. These results suggested that fibrinogen adsorbed on PSf membranes induced GPIIb/IIIa-mediated platelet activation and Mac-1 and αvβ3-mediated neutrophil activation, in proportion to the amount of adsorbed fibrinogen.

In Chapter II, I assessed the effects of two HD PSf membranes on the formation of platelet-neutrophil complexes *in vitro*. CX-U, which has high ability to activate cells, induced the formation of platelet-neutrophil complexes, and subsequent neutrophil activation. On the other hand, NV-U, which has slight or no ability to activate cells, caused neither the formation of platelet-neutrophil complexes nor subsequent neutrophil activation, even when co-incubated with platelets. These results suggested that varying platelet and neutrophil activation among HD membranes causes parallel

differences in the formation of platelet-neutrophil complexes.

This study has presented that a HD membrane with lower fibrinogen adsorption can reduce cell activation and platelet-neutrophil complexes formation during dialysis, which may lead to subsequent pathological conditions, such as microvascular inflammation and oxidative stress in HD patients. And it indicates that the membrane with lower fibrinogen absorption decrease the risk of development and progression of various HD-associated complications, such as accelerated atherosclerosis. In addition, this study has provided a new guidance for the development of biocompatible HD membranes to improve HD treatment.

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