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Endothelial cells on plasma-treated segmented-polyurethane

Adhesion strength, antithrombogenicity and cultivation in tubes

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When the surface of segmented-polyurethane (SPU), where endothelial cells are not capable of proliferating, is modified by plasma treatment, the adhesion and proliferation of bovine aortic endothelial cells (BAECs) can be drastically improved. The cells were capable of proliferating on the inner surface of a plasma-treated SPU-coated tube (length: 50 mm; inner diameter: 1.5 mm). When a steady flow shear stress of 9 Pa was applied to the cells proliferated on the modified SPU surface for 90 min, most cells did not detach from the surface. From an *in vitro* evaluation test of antithrombogenicity, the cell surface can be considered to provide an inert surface against thrombus formation and blood coagulation. From analyses of the plasma-treated SPU surface, it was suggested that the improvements in BAEC proliferation and adhesion after plasma treatment were due to the change in wettability of the surface. Data suggest that the plasma treatment would be useful for developing a small-calibre hybrid vascular graft.

1. Introduction

The primary failure modes of a small-calibre synthetic vascular graft are thrombus formation in the short term, and intimal hyperplasia, caused by compliance mismatch between the graft and host tissue, in the longer term. The use of an elastic polymer can overcome the latter failure in part. At present, however, no polymers can provide a sufficiently antithrombogenic surface for the substitution by themselves.

The pre-lining of the inner surface of a synthetic vascular graft with endothelial cells (ECs), called "hybridization", is one promising method. Many investigations have been undertaken to form the hybrid vascular graft using a clinically applicable tube made of such materials as polytetrafluoroethylene (PTFE) [1–3] and polyethylene terephthalate (PET) [4]. Segmented polyurethanes (SPUs) have been reported to have fairly good compliance [5], but they have not been used as a substrate of the hybrid vascular graft, because cells are not capable of confluently proliferating or strongly adhering on SPU surfaces.

It has been demonstrated that the cell attachment on polymer materials is influenced by physical and chemical surface structures, and can be improved by surface modifications [6]. In our recent studies, ion implantation, a technique used in the manufacture of semiconductors, has been shown to improve cell attachment and proliferation on an SPU surface [7]. It has been demonstrated that the improvement in cell adhesion on the SPU surface is also achieved by carbon deposition [8]. When the hybrid vascular graft is clinically utilized as a small-calibre artery substitution, its length should be 10 cm or more. In addition, a polymer sheet is not permitted to be formed into a tubular substrate after modifying its surface, because the seam can adversely affect the compliance of the hybrid vascular graft. Consequently, the inner surface of a longer tube material needs to be modified. However, the aforementioned two methods are of some disadvantage in modifying a middle portion of such a longer tubular substrate.

Plasma discharge treatment has been extensively used for modifying surfaces of polymer materials [9]. Plasma-treated polystyrene is widely used for cell culture, realizing good cell adhesion and proliferation on its surface. However, the plasma discharge treatment usually requires a fairly complex process. In a preliminary study, we found that bovine aortic endothelial cells (BAECs) proliferated to confluency on the surface of an SPU sheet simply treated with plasma made from air.

In this study, we tried to apply the simple treatment with air plasma to modification of the inner surface of a longer SPU tube, and investigated the feasibility of the combination of BAECs and SPU for a small-calibre hybrid vascular model. We modified the inner surface of a tubular SPU coating by plasma treatment. We cultivated BAECs on the plasma-treated surface in order to investigate whether the BAEC proliferation on it was sufficient for a small-calibre hybrid vascular graft. We also determined the resistivity of BAECs cultured on the surface of a plasma-treated plane SPU coating, to flow shear stress, and their anti-coagulability in order to evaluate whether plasma-treated SPU was applicable to the hybrid vascular model. In addition, the surface of the

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plasma-treated SPU is analysed in order to elucidate a mechanism of EC adherence on the plasma-treated SPU.

2. Materials and methods

2.1. Polymer

An SPU of KP-13 was used in this study. KP-13 contains 68% soft segment, 13% of which is poly-(ethylene oxide)-poly(dimethylsiloxane)-poly(ethylene oxide) [10]. It was a gift from Kaneka Corporation (Osaka, Japan). KP-13 was dissolved in tetrahydrofuran (THF; Wako Pure Chemical Industries Ltd., Osaka, Japan) at a concentration of 15 mg/ml.

2.2. Coating

(a) Glass tubes (50 mm in length, 1.5, 2 or 3 mm in inner diameter). The inner space of a tube was filled with the KP-13 solution, so that the whole inner surface of the glass tube was set in contact with the solution. Subsequently, the glass tube was left tilted so that the solution gradually ran out.

(b) Glass tubes with a flat closed end (30 mm in length, 10 mm in inner diameter): 0.5 ml of the KP-13 solution was poured into a tube, and the whole inner surface of the tube was made to contact the solution several times by tilting and rotating the tube. Subsequently, the excess solution was removed, and then the glass tube was left for several minutes to stand with the closed end up.

(c) Flat slide glass (76×26 mm) or glass petri dish (inner diameter: 28 mm). 1 ml of the KP-13 solution was uniformly spread on a pre-cleaned slide glass or glass petri dish.

Glasses (a), (b) and (c) were slowly dried in air at room temperature overnight to obtain a transparent coating.

2.3. Plasma treatment

Plasma treatment of the SPU surface was carried out with an ion coater (type IB-2; Eiko Engineering Co. Ltd., Ibaraki, Japan). This apparatus originally had two electrodes (diameter: 7 cm), opposed to each other at a distance of 3.5 cm in a vacuum chamber.

(a) SPU-coated glass tube (50 mm in length, 1.5, 2 or 3 mm in inner diameter). We made electrodes composed of a pair of stiff metal wires opposite each other at their ends (distance between the ends: 5 mm) in the vacuum chamber of the ion coater. The wire electrodes were connected to the original electrodes which were electrically insulated. They were put through an SPU-coated glass tube. A high tension of approximately 700 V (DC) was applied to the wire electrodes. The inner surface of the SPU-coated glass tube was treated by plasma generated between the wire electrodes.

(b) SPU-coated glass tube (30 mm in length, 10 mm in inner diameter). The wire electrodes connected to the electrically insulated original electrodes were disposed in parallel at a distance of 5 mm. They were put into an SPU-coated tube from its open end. A high tension of approximately 700 V (DC) was applied to the wire electrodes. The inner surface of the SPU-coated glass tube was treated by plasma generated between the wire electrodes.

(c) SPU-coated slide glass or glass petri dish. A sample was just placed on the original lower electrode. A high tension of approximately 700 V (DC) was applied to the electrodes in the etching mode (upper electrode: positive).

The high tension was applied for 5 min after the chamber pressure attained to 5 to 10 Pa. The ion current was kept at 4 to 5 mA throughout the treatment. The surface-modified SPU was thermally treated in dry air at 130 °C for 4 h, subsequently sterilized with UV irradiation (wavelengths: 254 and 366 nm) for 30 min before use.

2.4. Cell culture

Bovine aortic endothelial cells (BAECs) were isolated from a descending aorta using 0.1% collagenase (Worthington Biochemical Corporation, NJ, USA) in Dulbecco's phosphate buffered saline (PBS) by a method adapted from Jaffe *et al.* [11] and Schwartz [12]. The method has been described in detail elsewhere [13]. The isolated cells were incubated in RPMI 1640 medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 20% fetal bovine serum (FBS; Sanko Junyaku Co. Ltd., Tokyo, Japan) at 37 °C in 5% CO_2 atmosphere. The cells were identified as endothelial by their uptake of DiI-acetylated low-density lipoprotein (Biomedical Technologies Inc., MA, USA). BAECs were harvested with 0.25% trypsin (1 : 250, Difco Laboratories, Michigan, USA) in Ca, Mg free PBS (PBS (−)) containing 0.02% ethylenediaminetetraacetic acid disodium salt (EDTA; Wako Pure Chemical Industries Ltd.), and passaged in the medium supplemented with 10% FBS. BAECs were routinely used between passages 4 and 9.

On the inner surface of a KP-13 coated glass tube ((a) and (b) in Section 2.3) or of a bare glass tube, BAECs were cultured by a rotatory cultivation method, which is described in detail elsewhere [13]. The BAECs cultured on the inner surface of the glass tubes were observed with a phase contrast microscope (type IMT-2; Olympus Optical Co. Ltd., Tokyo, Japan). On the plane surface ((c) in 2.3), BAECs were cultured by such a method as currently employed for subculture.

2.5. Quantification of BAECs detached under flow shear stress

BAECs cultured on a surface-modified SPU film formed on a slide glass were exposed to laminar flow in a parallel plate flow chamber (Fig. 1). The lower plate of the chamber with two inlet–outlet slits (1×20 mm) is made of transparent polymethylmethacrylate (PMMA). The flow path was formed by inserting a silicone rubber gasket between the lower plate and an SPU-coated slide glass on which BAECs were confluently cultured. The thickness of the gasket used was 0.3, 0.5 or 1.0×10^{-3} m, defined as a height h of the

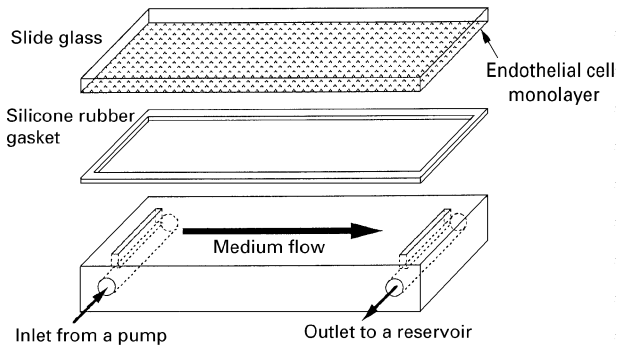

Figure 1 Schematic diagram of a parallel plate type flow chamber used for applying flow shear stress to endothelial cells (ECs) cultured on a modified segmented polyurethane (SPU) surface. A SPU-coated slide glass on which ECs were confluently cultured was mounted together with a silicone rubber gasket to a lower PMMA plate. The flow shear stress was imposed to the ECs by circulating culture medium maintained at 38 °C in a reservoir using a pump.

flow path. The BAECs-cultured slide glass was mounted in a PMMA fixture, which was fixed to the lower plate using eight screws. The width w and length l of the flow path were 22 and 72×10^{-3} m, respectively. RPMI 1640 supplemented with 1% FBS was introduced from a reservoir to the inlet of the chamber through a pump (type FJ; Haake Gebrueder, Berlin, Germany). The temperature of the flow medium was maintained at 38 °C in the reservoir. The volumetric flow rate $Q(\mathrm{m^3\,s^{-1}})$ was calculated from the time required to fill a 100 ml graduated cylinder. One-dimensional laminar flow can be obtained in the flow path. The wall shear stress τ_w (Pa) is calculated from

$$\tau_w = 6\mu Q/wh^2 \quad (1)$$

where μ is the medium viscosity (Pa s), which was set at 0.69×10^{-3} (viscosity of water at 37 °C) in this calculation [14, 15]. The flow chamber was placed on the stage of a phase contrast microscope (type EHT; Olympus Optical Co. Ltd.). BAECs exposed to flow shear stress were photographed at appropriate intervals. The number of adherent cells in an area of 0.16 $\mathrm{mm^2}$ (0.4×0.4 mm) was determined by counting cells in the micrograph.

2.6. Analysis of the antithrombogenicity of BAECs cultured on plasma-treated SPU surface

The coagulation process of a blood sample put in a vascular model tube composed of a glass tube (length: 30 mm, inner diameter: 10 mm; refer to Section 2.2. (b)), plasma-treated KP-13 coating and BAEC monolayer was monitored using a damped oscillation type rheometer, the structure of which is described in detail in previous papers [13, 16, 17]. The change in logarithmic damping factor (LDF) of the blood sample put in the vascular model tube can be sensitively detected by the rheometer. The LDF closely relates to the viscosity and/or viscoelasticity of the blood sample, decreasing with the increase in viscosity of the blood sample. Blood samples were prepared from blood taken from adult volunteers using trisodium citrate (final concentration: 0.38%) and the coagulation reaction was initiated by adding 85 µl of 0.25 M $CaCl_2$ to 1 ml of the sample.

2.7. Analysis of modified SPU surfaces

The water contact angle of modified KP-13 surfaces was measured with a CD-A contact angle meter (Kyowa Kaimenkagaku Co. Ltd., Tokyo, Japan).

XPS analysis of modified KP-13 surfaces was carried out with a VG ESCALAB MK II spectrometer using MgK_α irradiation (1253.6 eV). The applied source was operated at 15 kV and 10 mA. The base pressure of the analysis chamber was less than 10^{-7} Pa. The binding energy scale was calibrated using Au $4f_{7/2}$ (83.6 eV) and/or the C 1s spectrum (284.6 eV) of a characteristic component. The take-off angle was fixed at 90 degrees.

3. Results

Fig. 2 shows BAEC proliferation on the inner surface of a KP-13-coated glass tube having an inner diameter of 2 mm, after 5 days of seeding. The micrographs (a) represent BAECs cultured on the plasma-treated KP-13 surface. In order to compare the effect of plasma treatment with that of another treatment, BAECs cultured on a KP-13-coated glass tube (inner diameter: 2 mm) treated by ion-implantation (b) or carbon-deposition (c) are also shown. The methods for treating a surface of SPU by carbon-deposition and ion-implantation are described in detail elsewhere [7, 8]. Results of the BAEC proliferation in the glass tubes are summarized in Table I. The BAECs cultured on the bare glass tube proliferated to confluency everywhere, irrespective of the inner diameters of 1.5, 2 and 3 mm. BAECs confluently proliferated in the end portions of the modified KP-13 coating, irrespective of the three modification methods and of the inner diameters. In contrast, they did poorly or did not proliferate in the middle portion of the 2 and 1.5 mm diameter KP-13-coated tubes, except for the plasma-treated ones. (In a modified KP-13-coated glass tube having a larger inner diameter, such as the tube described in Section 2.3. (b), BAECs confluently proliferated everywhere, irrespective of the three modification methods.) The morphology of the BAECs proliferated to confluency was a typical cobblestone-like shape, indicating that they were normally cultured on the modified KP-13 surfaces.

In order to determine the adhesion strength of BAECs, we imposed a flow shear stress on a monolayer of BAECs confluently cultured on a substrate using a parallel plate type flow chamber. Fig. 3 shows phase contrast micrographs of BAECs cultured on a flat bare glass (a, b) and the plasma-treated KP-13 surface (c, d). The micrographs (a, c) represent BAECs before imposition of a flow shear stress. The micrograph (b) represents BAECs after imposition of a flow shear stress of 0.2 Pa for 15 min, and (d) after imposition of a flow shear stress of 5.5 Pa for 90 min. Most of the BAECs cultured on the flat bare glass were detached by imposing a flow shear stress of 0.2 Pa. In

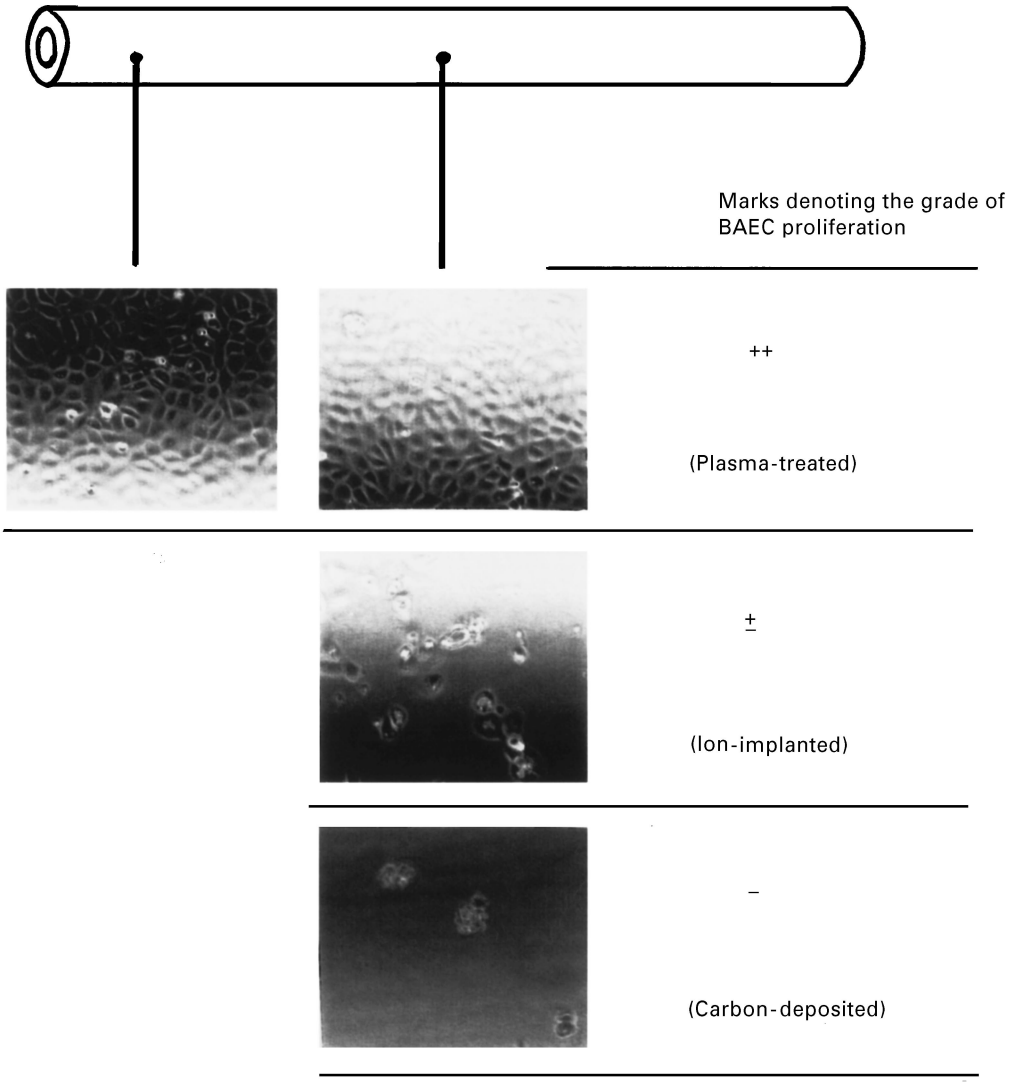

Figure 2 Phase contrast micrographs of bovine aortic endothelial cells (BAECs) on the inner surface of a KP-13 coated glass tube. The marks denoting the grade of BAEC proliferation are represented by phase contrast micrographs, and also used in Table I. Ne^+ ions having an energy of 150 keV were implanted at an area density of 10^{15} ions/cm^2. The pressure in the implantation chamber was 1.3 to 4.0×10^{-4} Pa. Carbon was deposited using a vacuum evaporator equipped with two carbon rods (diameter: 5 mm, purity: 99.999%). An electric current of 30 A was introduced to the carbon rods, located at a distance of 13 cm from a sample, for 5 to 10 s after the pressure in the vacuum chamber attained to 10^{-3} Pa (a) Plasma treated; (b) ion-implanted; (c) carbon deposited.

TABLE I BAEC proliferation on the inner surface of KP-13 coated glass tubes

ID (mm)	Non-treated		Plasma-treated		Carbon-deposited		Ion-implanted		Bare glass	
	end	middle	end	middle	end	middle	end	middle	end	middle
3	−	−	++	++	++	−	++	+	++	++
2	−	−	++	++	++	−	++	±	++	++
1.5	−	−	++	++	++	−	++	−	++	++

++: Proliferated to confluency; +: Fairly proliferated, but not to confluency; ±: Poorly proliferated; −: Not proliferated nor adhered.

contrast, almost no BAECs cultured on the plasma-treated KP-13 surface were detached after imposition of a flow shear stress of 5.5 Pa for 90 min, and 89 ± 3% (mean ± SD) of the initial retained after imposition of a flow shear stress of 9.0 Pa for 90 min.

In order to evaluate the antithrombogenicity of BAECs cultured on a plasma-treated KP-13 surface, we monitored the coagulation process of whole blood or platelet-free plasma (PFP; platelet count of less than 100/μl) put in a vascular model tube. For comparison, we also monitored that of whole blood or PFP put in a plasma-treated KP-13 coated tube without cultured BAECs. Fig. 4 shows changes in LDF of whole blood and PFP placed in the respective tubes. In the vascular model tube, PFP did not coagulate throughout the measurement period (> 120 min), and whole blood began to coagulate at about 30 min. In the plasma-treated KP-13-coated tube, in contrast, both whole blood and PFP began to coagulate in a few minutes.

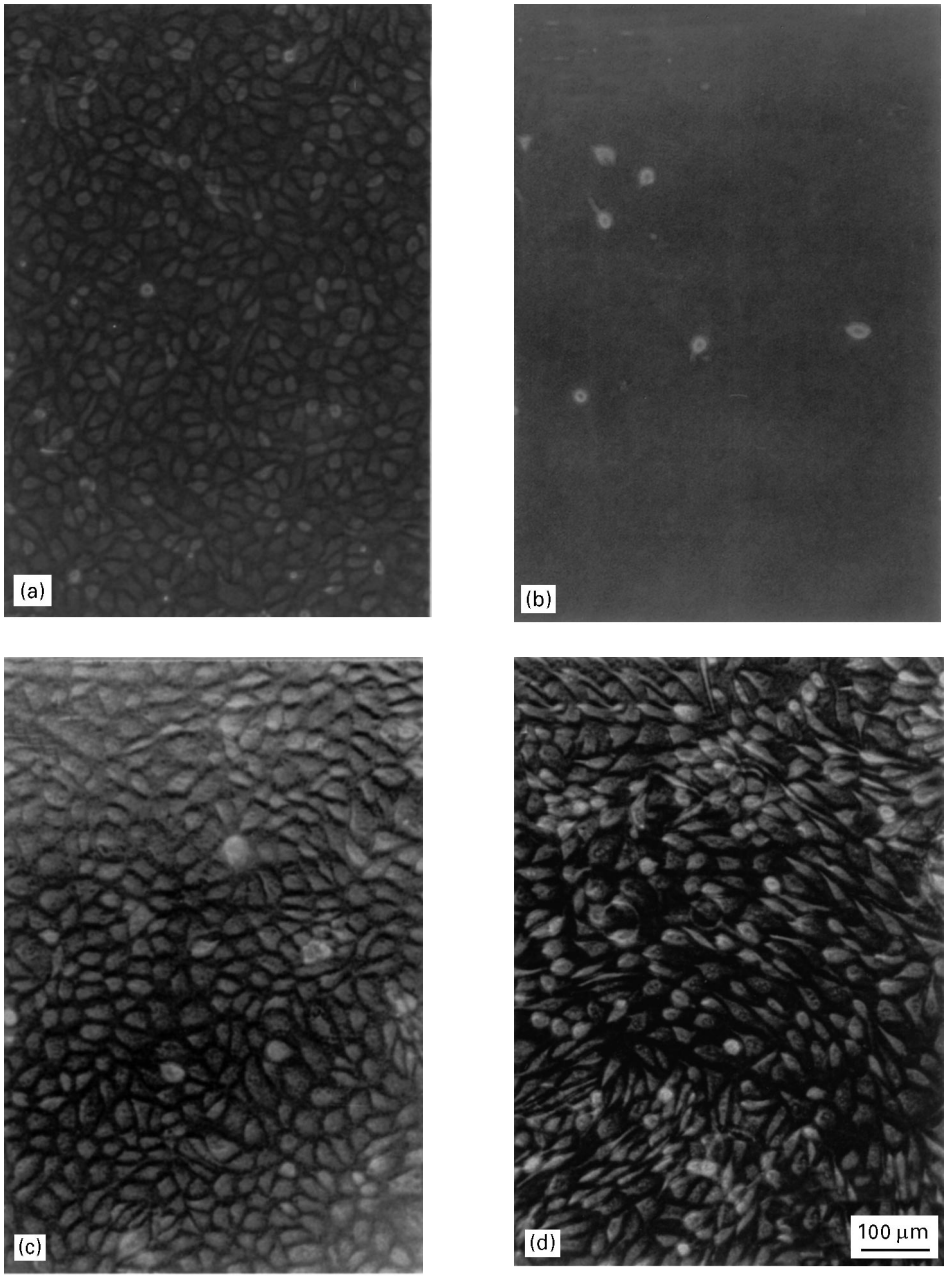

Figure 3 Phase contrast micrographs of bovine aortic endothelial cells (BAECs) cultured on the surface of bare glass or plasma-treated KP-13 after imposition of a flow shear stress. Bare glass: (a) initial; (b) 0.2 Pa for 15 min. Plasma-treated KP-13: (c) initial; (d) 5.5 Pa for 90 min.

We analysed plasma-treated surfaces of KP-13 to elucidate the mechanism of BAEC adhesion on the plasma-treated KP-13 surface. Fig. 5 shows a time-course change in water contact angle of the plasma-treated KP-13 surface. Non-treated KP-13 had a water contact angle of 98 degrees, which was very significantly decreased to approximately 20 degrees after plasma treatment. The water contact angle increased with time and reached 52 degrees after 24 h in air.

Overall X-ray photoelectron spectra of the non-treated and plasma-treated KP-13 surfaces are shown in Fig. 6. From the atomic composition quantified by XPS, the oxygen content was 21 atom % (C/O ratio: 3.1) in the non-treated KP-13 surface layer, increased to 40 atom % (C/O ratio: 1.1) after plasma treatment, and then decreased gradually to 32 at % (C/O ratio: 1.6) over 40 days.

4. Discussion

Vascular prosthesis substitution is one successful option for treating angiostenosis or arterial occlusion. When a small-calibre (3 mm or less) artery, such as a coronary, is by-passed by the substitution, a venous autograft is usually used, because artificial vascular grafts now in clinical use have insufficient antithrombogenicity for by-passing. When the inner surface of a hybrid vascular graft is coated with ECs, a shear stress generated by luminal blood flow is imposed on the ECs. The use of fibronectin in the formation of a polyurethane-based hybrid vascular graft has

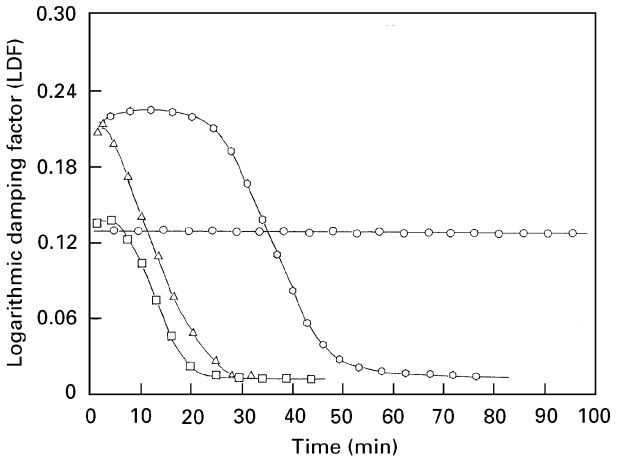

Figure 4 Changes in logarithmic damping factor (LDF) during coagulation of whole blood and platelet-free plasma (PFP) put in a glass tube with plasma-treated KP-13 coating, or vascular model tube composed of a glass tube, plasma-treated KP-13 coating and BAEC monolayer. Glass tube with plasma-treated KP-13 coating: (△) whole blood; (□) PFP. Vascular model tube: (⬡) whole blood; (○) PFP.

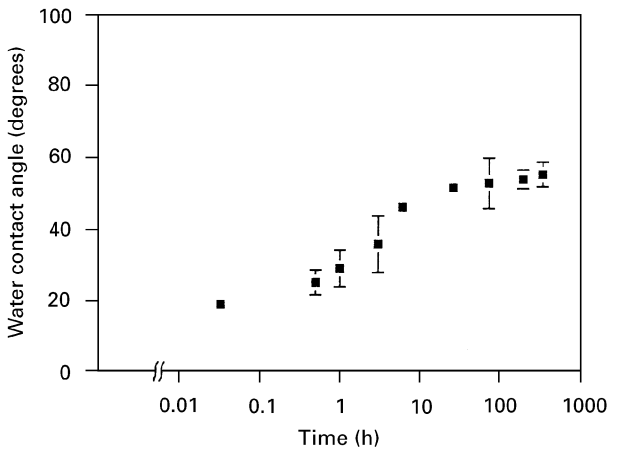

Figure 5 Time-course change in water contact angle of a plasma-treated KP-13 surface. The square mark denotes the average value of measurements at each time, and the bar denotes the standard deviation (SD) of the measurements ■ non-treated.

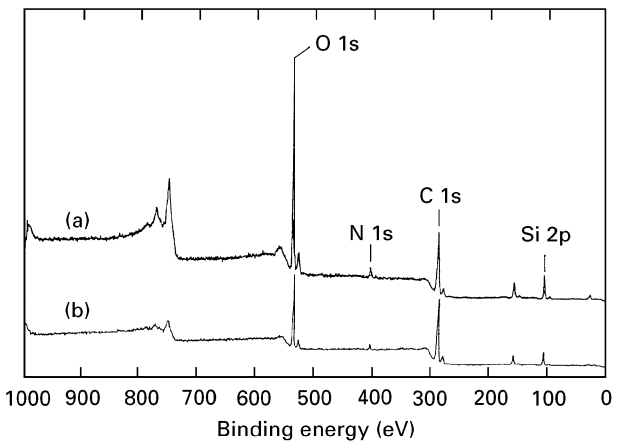

Figure 6 Overall X-ray photoelectron spectra (XPS) of KP-13 surfaces: (a) plasma-treated; (b) non-treated.

been reported to realize good EC proliferation to confluency, but to lead to about 50% loss of the initial EC covering after implantation for a week [3]. The loss of ECs probably arises from shear stress generated by luminal blood flow. The ECs of a hybrid vascular graft need to be strongly adherent on the substrate. Improved adherence of ECs to SPUs, which are fairly biocompatible, can improve the feasibility of the hybrid vascular graft.

As shown in Table I, good BAEC proliferation was observed on the middle portion of the inner surface of the plasma-treated KP-13-coated glass tube having an inner diameter of 1.5 mm and on a bare glass tube having the same inner diameter. However, BAECs did not adhere or proliferate in the middle portion of ion-implanted or carbon-deposited glass tubes with an inner diameter of 1.5 mm at all. In our method of ion implantation, ion beams were applied to the inner surface from the open end of a glass tube. The ion beams are supposed not to have sufficiently irradiated the middle portion of the inner surface for its modification. In our method of carbon deposition, vaporized carbon was also deposited on the inner surface from an open end. The vaporized carbon is supposed to be incapable of reaching the middle portion. These results indicate that ion implantation and carbon deposition are applicable to modifying the inner surface of a tube material having a diameter relatively large compared to its length, and the method of plasma treatment employed in this study is very effective for modifying the inner surface of a longer tubular material. This suggests that a monolayer of BAECs can be formed on the whole inner surface of a small-calibre SPU tube.

The flow shear rate of blood at the wall of a small-calibre artery has been found from calculation assuming laminar flow, to be more than 700 s^{-1} [17]. It has been reported that the viscosity of normal whole blood is 3 to 6 mPa s [18]. These results indicate that the ECs covering the inner surface of a vascular graft are required to be maintained under a flow shear stress of at least 2 Pa, preferably more, when the vascular graft is used for by-passing a small-calibre artery. When the flow shear stress under which almost all BAECs cultured were retained is defined as the adhesion strength of BAECs, the adhesion strength on plasma-treated KP-13 was at least 5.5 Pa (Table II). This suggests that they tolerate a flow shear stress equivalent to that generated on the wall of a small artery. Although the adhesion strengths of ECs on a substrate probably vary among the different sources, plasma-treated SPU can provide a surface applicable as the substrate of a hybrid vascular graft.

The antithrombogenic function of cultured BAECs have been reported to be altered by the substrate used [13]. In our recent studies, it has been shown that PFP (having no cellular components) placed in a simple vascular model tube composed of a glass tube

TABLE II Adhesion strength of BAECs (Pa)

Bare glass	<0.2
Plasma-treated KP-13	>5.5

and BAEC monolayer does not coagulate throughout a measuring period of over 120 min [19], indicating that the intrinsic and extrinsic coagulation pathways are not activated in this model tube [20]. It has also been shown that whole blood in the simple vascular model tube keeps its original fluidity for about 30 min under quasi-stagnant conditions *in vitro* [19]. The coagulation of whole blood described above was observed to occur in any inert tube, and reported to be mainly due to stagnant red blood cells existing in the blood, there probably being prevented in flowing blood [21]. This indicates that the simple vascular model tube is basically non-thrombogenic. As shown in Fig. 4, the bare surface of plasma-treated KP-13 was originally procoagulant. PFP and whole blood placed in a vascular model tube comprising a plasma-treated KP-13 coating also led to almost the same results as in the simple vascular model tube (Fig. 4). The procoagulant characteristic of the plasma-treated KP-13 surface was drastically changed into anti-coagulant by coating with BAECs, suggesting that the BAECs cultured on the plasma-treated KP-13 kept their antithrombogenic function.

The carbon-deposited KP-13 surface has been reported to have a water contact angle in the range 70 to 80 degrees [8]. The water contact angle of the plasma-treated KP-13 surface increased with time, and became more than 50 degrees after 24 h (Fig. 5). The intermediate hydrophilicity of polymer surfaces in the range of water contact angle between 50 and 80 degrees has been reported to induce good cell adhesion [22]. The change in cell adherence and proliferation on the SPU surfaces after plasma treatment is thought to be due to the change in hydrophilicity of the treated surfaces. XPS analysis of the plasma-treated KP-13 showed that the oxygen content in the surface layer markedly increased after plasma treatment (Fig. 6), and gradually decreased with time. Active groups including oxygen, such as carbonyl and carboxyl, have been reported to be introduced onto a polymer surface by O_2 gas plasma treatment [9]. The increase in hydrophilicity (decrease in water contact angle) after plasma treatment is thought to arise out of an increase in oxygen content in the surface layer.

Cell adhesive molecules, such as fibronectin, have been reported to play an important role in the attachment of cells to substrates [1, 15]. It has been reported that fibronectin can be easily adsorbed to the carbon-deposited KP-13 surface [8]. The intermediate hydrophilicity of the plasma-treated KP-13 surface is thought to facilitate the fibronectin adsorption onto them. The BAECs cultured on the plasma-treated SPU surface showed high trypsin resistivity (data not shown). Fibronectin molecules have been reported to be processed into crosslinked multimers by ECs, causing ECs to be resistive to trypsin-induced detachment. The processed fibronectin molecules are thought to bring about strong adhesion of ECs to extracellular matrices [23]. The high adhesion strength of BAECs on the plasma-treated KP-13 surface probably arises from fibronectin molecules which are strongly adsorbed onto the surface, subsequently multimerized by BAECs.

From the results of this study, it can be concluded that the air plasma treatment is of great advantage for modifying the inner surface of a long SPU tube, and plasma-treated SPU tubes are promising for a substrate of hybrid vascular grafts.

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