Transactions of the Materials Research Society of Japan 28 [2] 489-494 (2003)

Cell Induction Technique by Ion Irradiated Collagen for Development of a Small Diameter Artificial Graft

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Our previous study indicated He^+ ion implanted collagen grafts with a fluence of $1x10^{14}$ ions/cm^2 have excellent blood contacting properties and demonstrated a high anti-thrombogenicity and grafts patency. He^+ ion implanted collagen with a fluence of $1x10^{14}$ ions/cm^2 has simultaneously the two properties of anti-thrombogenicity and cell attachment. Biochemical and physico-chemical evaluation such as *in vitro* platelet adhesion tests, plasma protein adsorption, endothelial cell attachment test, and surface characterization were performed to investigate the mechanisms of antithrombogenicity of the surfaces. From these consequences, anti-thrombogenicity of the He^+ ion implanted collagen was caused by both the reduction of the plasma protein adsorption such as fibrinogen or von Willebrand factor and the destruction of the GFOGER sequences inside collagen by ion implantation. We think He^+ ion implanted collagen surfaces will be used widely as a base material in the medical fields.

Key words: ion beam, artificial graft, collagen, protein adsorption, antithrombogenicity.

1. INTRODUCTION

Several different approaches have been used to give vascular prosthetic materials an antithrombogenic luminal surface that would increase the patency of small internal diameter grafts. Currently available vascular prosthesis materials are hydrophobic and do not support endothelial cell growth[1] without a precoating with adhesive proteins such as fibronectin[2], vitronectin [3] or collagen [4], or surface modification such as plasma treatment [5]. Ion-implantation techniques are becoming popular as a versatile tool for modifying surface chemistries that improved the wettability [6], cell adhesion [7], and biocompatibility of polymer surfaces [8].

He^+ ion-implantation into inner surfaces of collagen (Type I)-coated ePTFE tubes with a length of 50 mm and an inner diameter of 3 mm were performed to develop hybrid type small diameter artificial vascular grafts. Those tubes were implanted into the dogs' carotid arteries and demonstrated 90% patency for 240 days. In addition endothelium at the graft surface was observed by the animal study [9].

It is generally accepted that the first event in the blood-biomaterial interaction is the adsorption of proteins onto the surface of the synthetic materials. Biomaterials absorbing the least amount of the plasma protein fibrinogen following exposure to blood will support less platelet adhesion and therefore exhibit less thrombogenicity. Furthermore, it has generally been accepted that the adhesion of platelets is promoted when proteins such as fibrinogen, von Willebrand factor (vWF) and fibronectin have been adsorbed to a material surface. On the other hand, platelet adhesion is reduced when pre-adsorbed albumin or high-density lipoprotein is present on the surfaces [10].

Furthermore, collagen interacts with platelets through at least two specific receptors. The platelet integrin collagen receptor $\alpha_2\beta_1$ is important for platelet binding to vascular endothelium and requires recognition of GFOGER, a specific peptide sequence in collagen.[11] Captured platelets are then activated through the platelet glycoprotein VI binding to a second collagen sequence GPO. Therefore, it is expected that these ligands may be destroyed by ion beams, so that platelets cannot be activated on the He^+-ion implanted collagen with a fluence of $1x10^{14}$ ions/cm^2.

The objective of the present work was to investigate the mechanisms of improving antithrombogenicity and promoting cell attachment of the He^+ ion implanted collagen surface with a fluence of $1x10^{14}$ ions/cm^2. In this report, we investigated in vitro endothelial cell attachment, plasma protein adsorption, in vitro and in vivo platelet adhesion to the He^+ ion implanted collagen surfaces as a function of fluence.

2. EXPERIMENTS

2.1 Ion implantation

Substrates used were polystyrene (PS) and ePTFE tubes with a length of 50mm. PS tubes with an inner diameter of 2 mm for acute studies and ePTFE tubes with 3mm for chronic studies were used. Ne^+ ion implantation into the PS tubes at energies of 150 keV with fluences of $3x10^{15}$ ions/cm^2 was performed as a pre-treatment of collagen coating. Those tubes' inner surfaces were coated with type I collagen (CELLGEN, Bovine dermis collagen, KOKEN co. Japan). After removing the surplus collagen, the tubes were dried under ambient conditions just in the Lab. Then He^+ ions were implanted into the inner surfaces at 150 keV with

fluences between $1x10^{13}$ and $1x10^{15}$ ions/cm^2. The ion beam current density was below 0.5 μA/cm^2 to prevent the substrates from heating.

2.2 *In vivo* acute animal study

In order to develop more competent artificial grafts, the effects of different ion fluences were studied under *in vivo* conditions. Eight Mongrel Dogs, each weigting about 20 kg, were operated under general anaesthesia. He^+ ion implanted collagen coated PS tube samples were performed with fluences of $5x10^{13}$, $1x10^{14}$ and $2x10^{14}$ ions/cm^2 and replace by dogs' femoral artery and vein up to 24 hours (Fig.1-a). After replacement, the specimens were scored for thrombus formation (Fig.1-b). From the data obtained, an anti-thrombogenic coefficient (AC) was calculated by using the following formula:

AC = Incubation time / Score

The AC shows that as the number increase, the substrate has anti-thrombogenicity.

2.3 *In vivo* chronic study

Mongrel Dogs, each weigting about 20 kg, were operated under general anaesthesia. A 5 cm segment of the carotid artery was replaced by the ePTFE tubes and ion implanted collagen coated ePTFE tubes. Those specimens were removed after 30 to 240 days replacement, and subjected to macroscopic, microscopic, and scanning electron microscopic observation.

All animal experimentation was approved by the School of Medicine, Teikyo University and Ethics Committee.

2.4 *In vitro* platelet adhesion

2.4.1 PRP platelet adhesion test

PRP ($1x10^5$ platelets/μl) was obtained from 3.8% sodium citrate solution-fresh human blood to prevent aggregation.

Then these samples were incubated on non-treated and ion-implanted surfaces at 37℃ for 30 min in a static system. After 30 min., the samples were fixed with 2% glutaraldehyde, dehydrated, and observed with a SEM.

2.4.2 *In vitro* washed platelet adhesion test

We performed a washed platelet test free from adhesive protein to examine the direct platelet adhesion to the collagen surface. Washed platelets were prepared according to the method of Katagiri et al.[12] at a final concentration of $1x10^5$ platelets/μl.

These samples were incubated on non-treated and ion-implanted collagen surfaces at 37℃ for 30 min in a static system. After 30min., these samples were fixed with 2% glutaraldehyde, dehydrated, and observed with a SEM. Platelets used contained 1% w/v bovine serum albumin at the final step of preparing a washed platelet solution to prevent non-specific interactions between platelets and substrates.

2.5 Endothelial cell culture

Bovine aortic endothelial cells (BAECs) were isolated from the descending aorta using 0.1% collagenase by a method adapted from Jaffe et al[13]. This cell suspension was placed on the ion implanted collagen coated dishes in medium (RPMI1640; Nissui Pharmaceutical Co.) supplemented with 10 % fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY). The initial number of the cells seeded was $1x10^4$ cells/cm^2. The cells were incubated for three days at 37 ℃ in 5 % CO_2 in humid atmosphere. The cell number was determined by counting after the cells were harvested by a short trypsin- EDTA treatment.

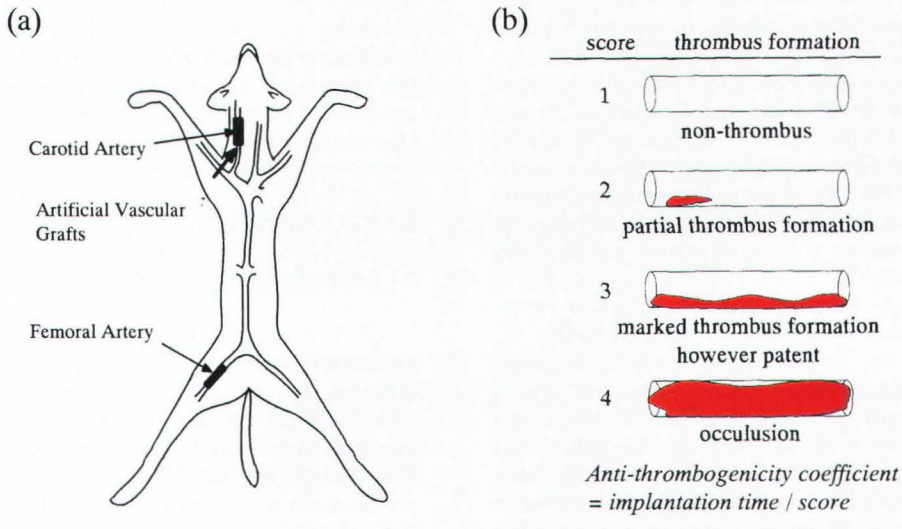

Fig. 1-a Position of implanted grafts in the dogs. Specimens were implanted into the carotid artery for chronic studies and the femoral artery and vein for acute studies of adult mongrel dogs

Fig. 1-b. Investigation of the anti-thrombogenic coefficient. After implantation tube samples were removed, the thrombogenic condition of the inner surface of these tubes were divided into four degree (score 1~4). Score 1; patent with no thrombus formation, score 2; patent with partial thrombus formation, score 3; patent with marked thrombus formation, score 4; occlusion.

2.6 Quantification of detached BAECs under flow shear stress

BAECs cultured on He^+ ion implanted collagen coated surfaces were exposed to laminar flow in a parallel plate flow chamber (Fig.2) in order to examine the effects of cell attachment strength to the modified surface.[14][15] One-dimensional laminar flow was obtained with the flow path (w= 22 mm), much larger than the gap height (h= 0.5 mm). For one-dimensional laminar flow, the wall shear stress t_w (dyne/cm^2) is

$$t_w = 6\mu Q/(wh^2)$$

where μ is the media viscosity (g/cm/s) (0.69 g/cm/s for distilled water at 37℃) and Q is the volumetric flow rate (cm^3/s). The shear stress used was 20 dyne/cm^2. The flow chamber was placed under a phase contrast microscope. BAECs exposed to flow shear stress were photographed at appropriate intervals. The number of cells was determined by counting cells in the micrographs.

Ion implantation was performed into the half side of the collagen coating plate. Bovine aorta endothelial cells were plated onto the samples in RPMI1640 medium with 10% FBS, and incubated at 37℃ for 5 days. When BAECs spread confluently on the specimens, they were exposed to laminar flow in a parallel plate flow chamber for 150 min. PBS (PH=7.3) was used for flow buffer.

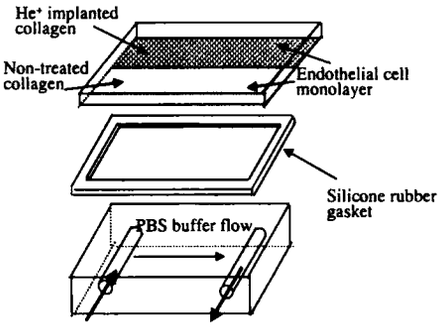

Fig. 2. Schematic of equipment of the flow experiment.
Schematic diagram of a parallel plate type flow chamber used for applying flow shear stress to endothelial cells cultured on the modified collagen surface.

2.7 Amino acid analysis of collagen

Collagen was coated on the dish samples and He^+ ion implantation were performed at 150 keV with fluences of $1x10^{13}$, $1x10^{14}$ and $1x10^{15}$ ions/cm^2. Non-implanted (control) and ion implanted dish samples were cut into 1x0.5 cm rectangles, and added 6N-HCl. Those specimens were decomposed with hydrochloride at the 110℃ temperature in vacuum. After the hydrolysis, the samples were dried up, dissolved in pure water and analyzed for their amino acid components (Model 835, Hitachi Co., Japan).

2.8 Immunoassay

To determine the adsorbed proteins at plasma-contacting surfaces, protein adsorption was evaluated by using a gold colloid labeled immunoassay.[16]

He^+ ion-implanted collagen tubes with fluences between $1x10^{13}$ and $1x10^{15}$ ions/cm^2 were cut into 1cm long. These samples were placed into 24-well tissue culture plates and contacted with human PFP for 120 min at 37 ℃. The PFP was removed, and the samples were rinsed three times with PBS. 2 ml of milk diluent was added to each plate, and the plates were kept at 4℃ overnight because it was necessary to block the non-specific reactive sites before applying antibodies to the specimens. After this procedure, these samples were rinsed three times with PBS, and incubated with the primary antibody [1wt% ovalbumin solution containing 2 μ g/ml of goat immunoglobulin of anti-human protein (Fg and vWf) for 60 min at 37 ℃. After rinsing three times with PBS, samples were fixed with 2% glutaraldehyde, then were labeled with the secondary anti-goat immunoglobulin with 10 nm colloidal gold (EM rabbit anti-goat IgG: 10nm gold, BBI international Co.). The gold colloid remaining after rinsing was enhanced to 200-300 nm with silver (Silver enhancer kit, Sigma). The membrane was freeze-dried and observed with a scanning electron microscope (FESEM, JED-6330F, JEOL, Japan).

3. RESULTS AND DISCUSSION

3.1 Acute animal study

Fig.3 shows the anti-thrombogenic coefficient of He^+ ion implanted collagen coated PS tubes with fluences of $5x10^{13}$, $1x10^{14}$ and $2x10^{14}$ ions/cm^2 and control collagen coated PS tubes (n=16～20). As a result of *in vivo* study, He^+ ion implanted grafts with fluences of $5x10^{13}$ and $2x10^{14}$ ions/cm^2 were occluded and platelets were not activated on the He^+ ion implanted collagen for a fluence of $1x10^{14}$ ions/cm^2. From these findings it was concluded that the optimal fluence for developing small artificial grafts was $1x10^{14}$ ions/cm^2.

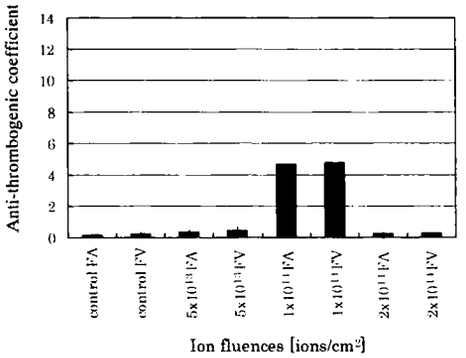

Fig. 3 Anti-thrombogenic coefficient by acute animal study
Specimens used were non-implanted, He^+ ion implanted collagen coated PS tubes, and implanted into 11 mongrel dogs' femoral artery (FA) and vein (FV) up to 24 h. After implantation, the specimens were scored for thrombus formation and calculated anti-thrombogenic coefficient (AC). The AC shows that as the number increase, the substrate has blood compatibility.

3.2 *In vivo* chronic study

Table 1 shows the graft patency of He^+ ion implanted collagen coated ePTFE grafts with a fluence of $1x10^{14}$

ions/cm^2 and non-treated ePTFE grafts. Those grafts were exposed to blood flow up to 240 days. More than 90 % of the He^+ ion implanted grafts were patent. On the contrary, non-treated ePTFE grafts were occluded within 10 days. Fig.4 shows the inner surface of the grafts (a, b) after implantation into dog's carotid artery for 90 days. He^+ ion implanted grafts demonstrated a high anti-thrombogenicity and patency.

Table 1.
Graft patency of animal chronic study

Control (ePTFE)[a]	
3 day	Occluded
10day	Occluded
ePTFE+ Ne^+ (150 keV,3x10^{15} ions/cm^2) + collagen+He^+(150keV,1x10^{14}ions/cm^2)[b]	
17day	Patent
30day	Patent
30day	Patent
90day	Patent
90day	Patent
90day	Patent
90day	Occluded
120day	Patent
180day	Patent
180day	Patent
180day	Patent
180day	Patent
240day	Patent

[a]Inner diameter, 3mm
[b]The animal used were adult mongrel dogs (♂) whose average weight was about 20 kg. He^+ ion implanted collagen coating ePTFE grafts with a fluence of 1x10 ^{14} ions/cm^2 were replaced by the carotid artery.

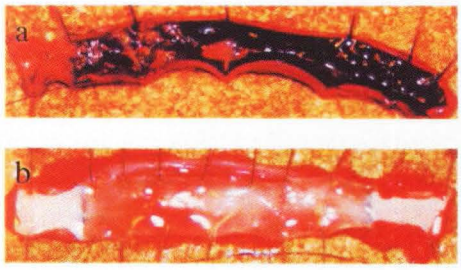

Fig.4. Animal Chronic study
Inner surface of non-ion implanted ePTFE (a) and He^+ ion implanted with a fluence of 1x10^{14} ions/cm^2 collagen coated ePTFE tubes implanted into carotid artery for 90 days (b).

3.3 *In Vitro* platelet adhesion

Fig. 5 (a-e) depicts *in vitro* platelet adhesion using Ca^{2+} re-added PRP for 30 mins. *In vitro* platelet adhesion was inhibited on the He^+ ion-implanted collagen-coated sample with a fluence of 1x10^{14} ions/cm^2 and maintained the same shape after 30 mins. In contrast, platelets activated on the untreated collagen surface and ion-implanted collagen with fluences of 1x10^{13}, 1x10^{15} and 1x10^{16} ions/cm^2; a fibrin network was observed on these platelets' activated surfaces.

Fig. 5 (f-j) illustrates platelet adhesion using washed platelets for 30 mins in 1% BSA. After the exclusion of the non-spesific interactions, the platelet activation was limited in untreated collagen and He^+ ion-implanted collagen with a fluence of 1x10^{14} ions/cm^2. Platelet adhesion was inhibited on the He^+ ion-implanted collagen with a fluence of 1x10^{13} ions/cm^2 and over a fluence of 1x10^{15} ions/cm^2. In particular, no platlets were observed on the He^+ ion-implanted collagen with a fluence of 1x10^{16} ions/cm^2. These results indicated that there were no ligands such as GFOGER on the He^+ ion-implanted collagen-coated dishes over a fluence of 1x10^{13} ions/cm^2 owing to the ion-beam destruction of GFOGER. However, washed platelets were activated on the 1x10^{14} ions/cm^2 implanted collagen. As a result, we expected that a functional group that the platelet could react with might be created by ion-implantation and that these functional groups might be blocked by plasma protein, resulting in antithrombogenicity being obtained. And it was expected that platelet activation over a fluence of 1x10^{15} ions/cm^2 was caused by adsorbed proteins.

3.4 Endothelial cell growth

Fig. 6. shows the endothelial cell growth curves of non-implanted and He^+ implanted collagen dishes with fluences of 1x10^{13}, 1x10^{14}, 1x10^{15} and 1x10^{16} ions/cm^2 for five days. Attachment and spreading of BAECs onto both ion implanted and non-implanted regions were observed. The rate of cell growth decreased with

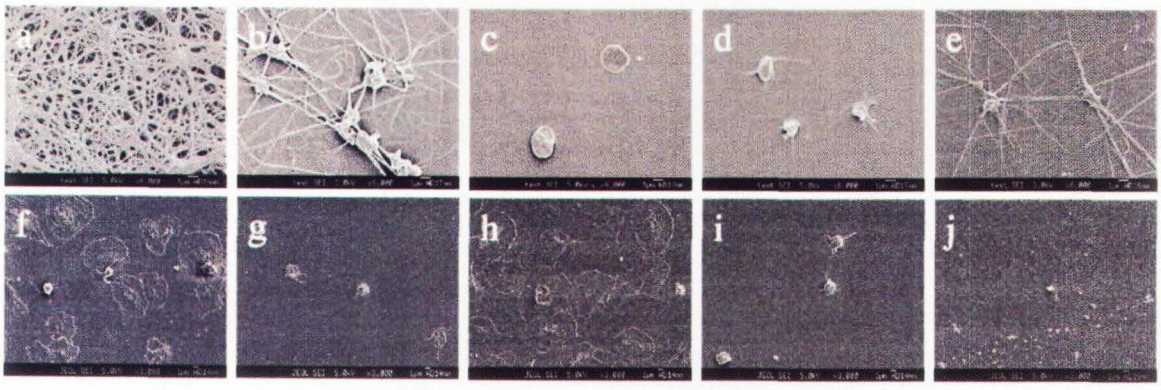

Fig. 5 SEM observation of the PRP platelet adhesion (a-e) and washed platelet adhesion (f-j) onto the untreated collagen (a, f) and the He^+ ion-implanted collagen with fluences of 1x10^{13}(b, g), 1x10^{14}(c, h), 1x10^{15}(d, i), and 1x10^{16} (e, j) ions/cm^2.

increasing ion fluence, however endothelial cell growth could still be observed for an ion fluence of $1x10^{14}$ ions/cm^2.

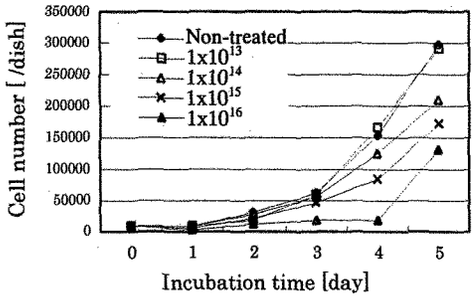

Fig. 6. Cell growth curves of non-implanted and He^+ implanted collagen dishes with fluences of $1x10^{13}$, $1x10^{14}$, $1x10^{15}$, and $1x10^{16}$ ions/cm^2.

3.5 Quantification of BAECs detached under flow shear stress

In order to determine the attachment strength of BAECs, flow shear stress was imposed on the layer of BAECs confluently cultured on He^+ ion implanted collagen surfaces with a fluence of $1x10^{14}$ ions/cm^2 using a parallel plate type flow chamber. Fig. 7 shows number of the cells on the non-implanted and He^+ ion implanted collagen under flow shear stress as a function of time. The difference between non-implanted and ion implanted collagen cannot be obtained under15 min flow shear stress. The attachment strength of the cell on ion-implanted collagen increased from 30 min flow shear stress as a comparison of non-implanted.

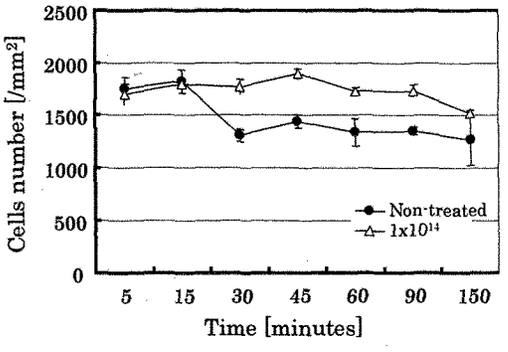

Fig.7. Quantification of BAECs detached under flow shear stress.
The ordinate shows the cell number per unit area of the photographs taken by a light microscope for 150 min.

3.6 Amino acid composition

Fig.8 shows the total protein of collagen (Type I) before and after ion implantation. The total amount of proteins decreased with increasing fluence. On the surface of the He^+ ion implanted collagen with a fluence of $1x10^{14}$ ions/cm^2 proteins still exist. However, little protein can be detected on ion implanted collagen with $1x10^{15}$ ions/cm^2, and the surface consists mainly of carbonized polymer. About 80% of the amino acids of collagen can be detected on collagen surface implanted with $1x10^{14}$ ions/cm^2. Totally, we concluded there is still exist the character of the collagen on the He^+ ion implanted with a fluence of $1x10^{14}$ ions/cm^2.

Fig.9 shows the amino acid composition. Type I collagen consist of about 20 amino acid. There were many ligands corresponds with cell membrane in the collagen. The ligand which recognized endothelial cell is Arg-Gly-Asp-(RGD) sequence. There is no Arginine acid on the He^+ ion implanted collagen with a fluence of $1x10^{15}$ ions/cm^2. Consequently, no-ligand such as RGD induced the cell spreading onto He^+ ion implanted collagen with a fluence of $1x10^{15}$ ions/cm^2.

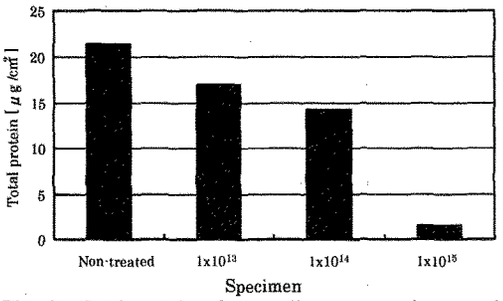

Fig. 8. Total protein of the collagen per unit area of non-implanted and He^+ ion implanted collagen surfaces with fluences of $1x10^{13}$, $1x10^{14}$ and $1x10^{15}$ ions/cm^2.

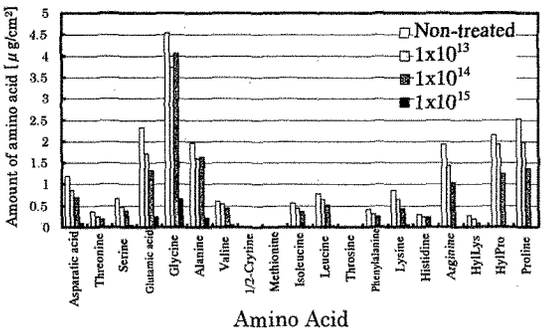

Fig. 9. The amount of the amino acid of non-implanted and He^+ ion implanted collagen surfaces with fluences of $1x10^{13}$, $1x10^{14}$ and $1x10^{15}$ ions/cm^2.

3.7 Immunoassay

The two-dimensional distribution of proteins (Fg and vWf) adsorbed on the He^+ ion-implanted collagen tubes' surfaces with fluences between $1x10^{13}$ and $1x10^{15}$ ions/cm^2 was determined. Fig. 10 shows the SEM photographs of the protein adsorption pattern on these samples. A considerable amount of protein adsorption was observed on the untreated collagen surface. The minimum adsorption was observed on the $1x10^{14}$ ions/cm^2 implantation surface. The adsorbed protein pattern was thus increased above the $1x10^{15}$ ions/cm^2 ion implantation. These tendencies correspond exactly to protein adsorption in a single solution.[17] We concluded that a low degree of protein adsorption reduced platelet adhesion. Collagen surfaces were carbonized over $1x10^{15}$ ions/cm^2 ion implantation, and a lot of adhesive protein such as vWF, fibrinogen or fibronectin[18] were

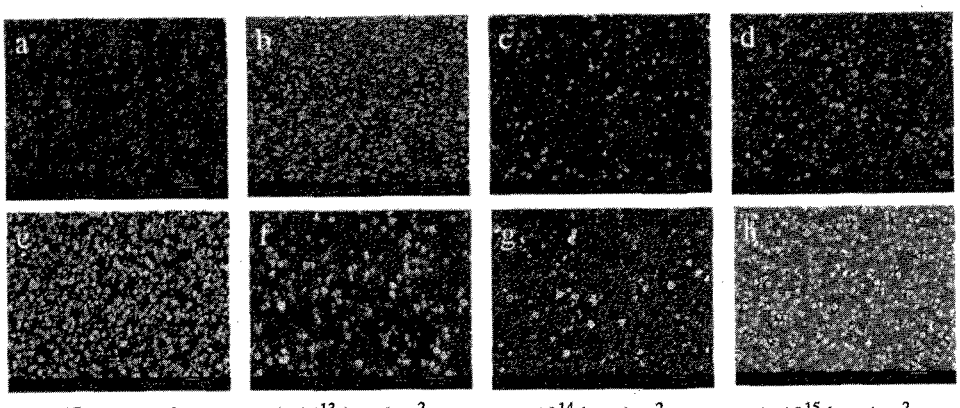

Fig.10. Protein adsorbed at the plasma-contacting surface of He^+ ion implanted collagen after 120 min adsorption from human plasma. Adsorption of vWF (a-d) and fibrinogen (e-h) at the plasma-contacting surface was detected by immunogold labeling technique.

absorbed onto these carbonized surfaces. As a consequence, platelets were activated over $1x10^{15}$ ions/cm^2 ion implanted collagen surfaces.

4. CONCLUSION

Adsorption of Fg and vWF from plasma onto a polymer surface promotes the adhesion of platelets. We therefore concluded that a low degree of protein adsorption reduced platelet adhesion on the He^+ ion-implanted collagen surfaces with a fluence of $1x10^{14}$ ions/cm^2. The results of the washed platelet adhesion test indicated that functional groups with which platelets can react might be created by He^+ ion implantation on $1x10^{14}$ ions/cm^2 implanted collagen and that these functional groups might be blocked by plasma proteins, resulting in antithrombogenicity being obtained. As a result, we speculated that antithrombogenicity of He^+ ion-implanted collagen with a fluence of $1x10^{14}$ ions/cm^2 was caused by both destruction of GFOGER sequences and plasma protein adsorption.

Chemical structure of collagen was decomposed and cell attachment property decreased as fluence increased. However, cell attachment strength was increased up to $1x10^{14}$ ions/cm^2. Ion-implantation also induces atomic mixing effects on the boundary between two layers. The phenomena were expected to apply to the ion-implantation into collagen-coated surface. It is concluded that ion implantation may produce new chemical bonds between collagen and substrate and it was thought that cell attachment strength increased owing to ion beam mixing effects.

The results from *in vitro* studies indicate that He^+ ion implantation into collagen with a fluence of $1x10^{14}$ ions/cm^2 left endothelial cell attachment property and decreases platelet adhesion. He^+ ion implanted collagen surfaces will be used widely as a base material in the medical fields.

References

[1] Pratt KJ, Jarrel BE, Williams SK, Carabasi RA, Rupnik MA, Hubbard FA. *J Vasc Surg*;7 (1988) 591-599.

[2] Schneider PA, Hanson SR. Price TM, Harker LA. *J Vasc Surg*; 8 (1988) 229-235.

[3] Steele JG, Johonson G, McFarland C, Dalton BA, Gengenbach TR, Chatelier RC, Underwood PA, Griesser HJ. *J Biomater Sci, Polym Edn*; 6 (1994) 511-532.

[4] Thomson GJL, Vohra RK, Carr MH, Walker MG. *Surgery*;109 (1991):20-27

[5] Kaibara M, Iwata H, Wada H, Kawamoto Y, Iwaki M, Suzuki Y. *J Biomed Mat Res*; 31 (1996): 429-435.

[6] Suzuki Y, Kusakabe M, Iwaki M. *Nucl Instr and Meth*; B91 (1994): 584-587.

[7] Suzuki Y, Kusakabe M, Lee JS, Kaibara M, Iwaki M, Sasabe H. *Nucl Instr and Meth;B* 65 (1992): 142-147.

[8] Suzuki Y, Kusakabe M, Akiba H, Kusakabe K, Iwaki M. *Nucl Instr and Meth; B* 59/60 (1991): 698-704.

[9] Suzuki Y, Iwata H, Nakao A, Iwaki M, Kaibara M, Sasabe H, Kaneko S, Nakajima H, Kusakabe M. *Nucl Instr and Meth; B* 127/128 (1997): 1019-1022.

[10] Lambrecht LK, Young BR, Stafford RE, Albrecht RM, Mosher DF, Cooper SL. *Thromb Res*; 41 (1986): 99-117

[11]. C. Graham Knight, Laurence F. Morton, Anthony R. Peachey, Danny S. Tuckwell, Richard W. Farndale, and Michael J. Barnes. *J Biol Chem.* , vol275, No.1, (2000): 35-40

[12] Katagiri Y, Hayashi Y, Yamamoto K, Tanoue K, Kosaki G and Yamazaki H. *Thromb Haemostas.*, 63, (1990): 122-126

[13] Jaffe EA, Nochman RL, Becker CG, Minick CR. *Clin Invest* 52 (1973) 2745-2756

[14] Lawrence MB, McIntire LV, and Eskin SG. *Blood* 70 (1987) 1284-1290.

[15] E.Sirois, J.Charara, J.Ruel, J.C.Dussalt, P.Gagnon, C.J.Doillon. *Biomaterials* 19 (1998) 1925-1934

[16] Y. Iwasaki, N. Nakabayashi, K. Ishihara. *J. Biomater. Sci., Polym. Ed.* 10 (1999) 513-529

[17] Kurotobi K, Kaibara M, Suzuki Y, Iwaki M, Nakajima H, Kaneko S. *Colloids and Surfaces B: Biointerfaces*; 19 (2000):227-235.

[18] Kurotobi K, Kaibara M, Suzuki Y, Iwaki M, Nakajima H. *Nucl Instr and Meth*; B175-177 (2001): 791-796.

(Received December 21, 2002; Accepted March 26, 2003)