ADSORPTION OF HUMAN RECOMBINANT FIBRONECTIN TO TITANIUM *IN VITRO*

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Statement of problem. Fibronectin mediates its biological effects by binding to integrins on cell membranes through a consensus site including the Arg-Gly-Asp (RGD) sequence within tenth type III module.

Purpose. The purpose of our study was to investigate the adsorption affinity of human recombinant fibronectin peptide (hFNIII 9-10) to titanium and to investigate the effect of the surrounding ionic composition on the adsorption process.

Material and methods. As for evaluating the affinity of hFNIII9-10 to Ti, titanium disks were incubated in 40, 80 and 120 μg/ml hFNIII9-10 solution at 37℃ overnight, repectively. As for evaluating the effect of surrounding ionic concentration, hFNIII9-10 was dissolved in distilled water, phosphate buffered saline and RPMI 1640. Optical density (O.D.) was measured in ELISA reader.

Results. The results were as follows;

1. The adsorption of hFNIII 9-10 showed significantly highest mean optical density (O.D.) value in 80 μg/ml.
2. The difference of ionic composition in DW, PBS and RPMI did not influence the adsorption amount of hFNIII 9-10.

Key Words

Fibronectin, Titanium

Titanium (Ti) has been widely used in dental and orthopedic fields as one of famous biomaterials. The success of Ti implants is related to their chemical interactions with the surrounding tissue and fluids. Upon exposure of Ti to oxygen, a highly stable oxide, TiO_2, range of 30～100Å thick, is formed on its surface. Owing to its high

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dielectric constant properties, titanium oxide (TiO_2) undergoes further modifications upon binding to various surrounding ions. These may include calcium, phosphate, and hydrogen, and result in the generation of OH^- radicals in the oxide. The oxide layer may adsorb macromolecules from the implant vicinity, including connective tissue components and proteins.[1]

Fibronectin (Fn) is a major extracellular matrix (ECM) component used by many cell types as a substrate for attachment.[2] Fn mediates its biological effects by binding to integrins on cell membranes through a consensus site including the Arg-Gly-Asp (RGD) sequence within tenth type III module.[3] A short sequence Pro-His-Ser-Arg-Asn (PHSRN) has also been identified as a synergistic motif within ninth type III module for binding to $\alpha_5\beta_1$ integrin.[4] Human recombinant fibronectin peptide (hFNIII 9-10), used in this study, is composed of those of nine and tenth type III module. (Fig. 1)

Suggested mechanisms of protein adsorption to biomedical polymers include hydrophobic interaction,[5-7] ligand specificity,[8] and electrostatic forces.[5] Several studies found that the adsorption of proteins,[9] connective tissue components such as the glycosaminoglycan chondroitin-sulrate,[10] human serum albumin and immunoglobulin G (Ig G) to Ti is affected by the presence of cations, such as Ca^{2+}.

The purpose of our study was to investigate adsorption affinity of hFNIII9-10 to Ti and the effect of the surrounding ionic composition on the adsorption process.

MATERIAL AND METHODS

Preparation of Ti disks

Commercially pure machined titanium disks (Grade 4) were provided by Daidon steel co., Korea. No polishing procedures were performed on the surfaces of disks. The disks were 25mm in diameter and 1mm in thickness. All disks were ultrasonically de-greased in trichloroethylen for 15 min followed by soaking in 70% ethanol for 10 min three times and were placed in distilled water overnight. The machined samples were dried and ethylene oxide sterilized.

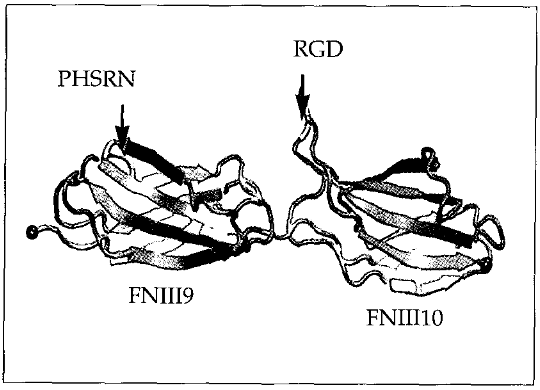

Fig. 1. Crystal structure of hFNIII9-10. The sequences of RGD and PHSRN are indicated by arrow. The image was prepared from the X-ray crystallographic coordinates taken from the molecular modeling database.

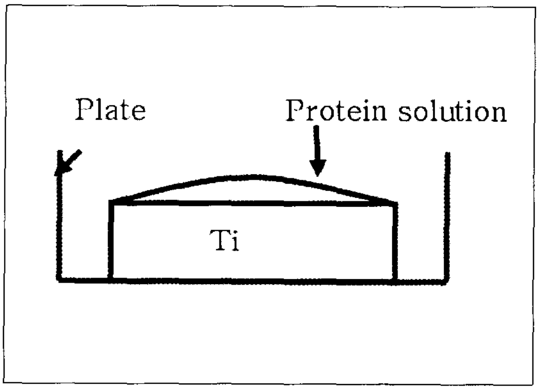

Fig. 2. Schematic drawing showing how protein suspension was placed on Ti surfaces during the study.

hFNIII9-10 adsorption to Ti

Construction of expression plasmids and purification of hFNIII9-10 were done in the same manner as those performed in the experiment of Jang et al.[11]

For the affinity of hFNIII9-10 to Ti, titanium disks were incubated in 40,80 and 120 μg/ml hFNIII9-10 solution at 37℃ overnight, repectively.

For evaluating the effect of surrounding ionic concentration, hFNIII9-10 was dissolved in distilled water, phosphate buffered saline (PBS, pH 7.4) (Gibsco BRL, Grand Island, NY), 1X RPMI 1640 and 2X RPMI (Gibsco BRL, Grand Island, NY). Each titanium disk in single plate was incubated in 1500μl 40 μg/ml recombinant hFNIII9-10.

Fig. 1 shows how protein suspension was placed on Ti surfaces. The samples were rinsed with PBS, incubated in His probe (H-3) antibody (Santa Cruz, biotechnology, CA), washed, and incubated in One step Turbo TMB ELISA (Pierce), which is substrate solution for ELISA. Optical density (O.D.) was measured at 450nm in ELISA reader (PowerWave X340-1) after adding 2M H_2SO_4 stopping solution.

Statistical analysis

All experiments were performed in a triplicate manner. The ANOVA test was used for statistical analysis of hFNIII9-10 adsorption to Ti disks. The level of significance was p <0.05

RESULTS

Adsorption affinity of hFNIII9-10 to Ti

hFNIII9-10 adsorbed almost two times higher in 80, 120 μg/ml than in 40 μg/ml, which was statistically significant. However, there was no significant difference in adsorption in 80 and 120 μg/ml which means fibronectin peptide adsorption is not proportional to the concentration.

Effect of the surrounding ionic composition on the adsorption process

There was no significant difference in fibronectin adsorption among groups. Although PBS group exhibited highest mean O.D. value and RPMI group exhibited lowest value, the difference was not significant.

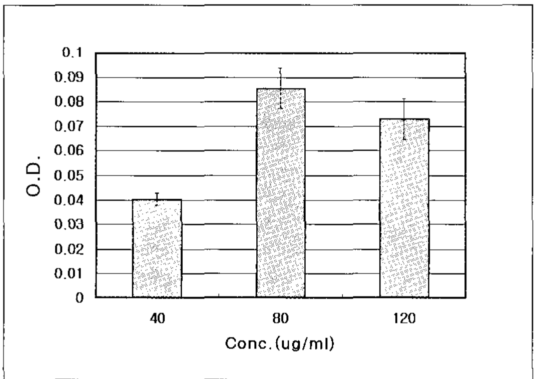

Fig. 3. Adsorption of hFNIII 9-10 in different concentration. Results are given as absorbancy (O.D.) values (mean ± standard deviation).

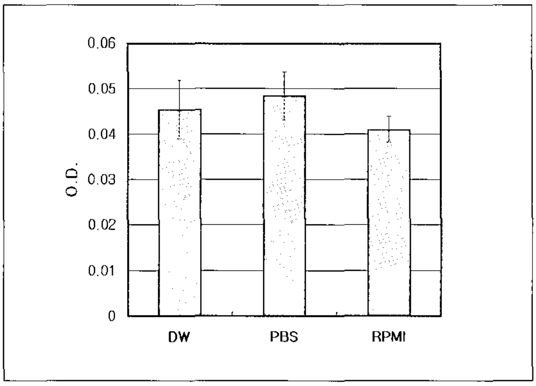

Fig. 4. Adsorption of hFNIII 9-10 dissolved in various solution. Results are given as absorbancy (O.D.) values (mean ± standard deviation).
DW= distilled water; PBS=phosphate buffered saline; RPMI=RPMI 1640 media

Table I. Ingredient of PBS and RPMI 1640 media

A. Phosphate-Buffered Saline (PBS)

Component	*1X liquid (mg/L)*
Inorganic salts;	
KH_2PO_4	144
NaCl	900
$Na_2HPO_4 \cdot 7H_2O$	795

B. RPMI 1640 Media

Component	*1X liquid (mg/L)*	*Component*	*1X liquid (mg/L)*	*Component*	*1X liquid (mg/L)*
Inorganic salts		L-Glutamic Acid	20.00	**Vitamine**	
$Ca(NO_3)_2 \cdot 4H_2O$	100.00	L-Glutamine	300.00	Biotin	0.20
KCl	400.00	Glycine	10.00	D-Ca Pantothenate	0.25
$MgSO_4$ (anhyd.)	48.84	L-Histidine (free base)	15.00	Choline Chloride	3.00
NaCl	5850.00	L-Hydroxyproline	20.00	Folic acid	1.00
Na_2HPO_4 (anhyd.)	800.00	L-Isoleucine	50.00	i-inocitol	35.00
Other components		L-leucine	50.00	Niacinamide	1.00
D-glucose	2000.00	L-Lysine · HCl	40.00	Para-aminobenzoic Acid	1.00
Glutathione reduced)	1.00	L-Methionine	15.00	Pyridoxine HCl	1.00
HEPES	5958.00	L-Phenylalanine	15.00	Riboflavin	0.20
Phenol Red	5.00	L-Proline	20.00	Thiamine HCl	1.00
Amino acids		L-Serine	30.00	Vitamin B_{12}	0.005
L-Arginine	200.00	L-Threonine	20.00		
L-Asparagine (free base)	50.00	L-Tryptophan	5.00		
L-Aspartic acid	20.00	$L\text{-Tyrosine} \cdot 2Na \cdot 2H_2O$	29.00		
L-Cystein · 2HCl	65.00	L-Valine	20.00		

DISCUSSION

During implant installation, plasma proteins and cells are seen at the blood- material interface after 5 seconds of exposure.[12] It has been reported that the titanium oxide plays an important role in the adsorption of proteins to implants.[1] The amphoteric characteristics of titanium oxide that resulted from surface hydroxy[1] groups were reported to change its surface zeta potential as a result of the changes in pH.[13,14] The isoelectric point of titanium oxide was reported to be 4.0-6.2.[15] At pH of 7.4, the oxide' s anionic character is reported to attract a variety of cations.[15,16] The cell adhesion is increased by the pre-adsorbed protein. The biologic effect depends on RGD sequence of extracellular protein rather than its types of protein.[17]

In this study, adsorption of hFNIII 9-10 showed highest value in the concentration of 80 $\mu g/ml$. In 120 $\mu g/ml$, although the concentration was higher than that of 80 $\mu g/ml$, O.D value was lower. From these results, we were able to interpret that maximum adsorption of hFNIII 9-10 in 10mm diameter titanium disk occurred in around 80 $\mu g/ml$. The pattern of fibronectin adsorption suggested that the fibronectin adsorption on titanium surface was limited to the availability of binding surfaces.

Similar protein adsorption kinetics patterns were observed in other studies. Klinger et al.[18] reported human albumin adsorbed continuously and reached a plateau, following a unilayer adsorption process in the given concentration. Protein adsorption over time also appeared increase protein adsorption occurring at steady state after sufficient time. According to Goldstein and DiMilla,[19] a maximum and a minimum value for a one protein monolayer coverage depends on orientation of fibronectin molecules. The maximum value occurs in a close -packed (side-up) arrangement and the minimum value does in a random sequential adsorption (side-down) with no overlap between protein molecules. Fibronectin conformation also depends on soaking concentration. At a low concentration, fibronectin may adsorb predominantly in a side-down conformation that is less favorable for mediating cell attachment.

Several studies reported that surronding ion concentration and other serum proteins may serve as a one of contributing factors in protein adsorption.[9,10,18] Positive correlation was observed between the presence of diavalent cations and the degree of albumin adsorption, and Ca^{2+} or Mg^{2+} takes part in as bridging agents in the adsorption process. However, the data of our study showed conflicting results. Compared to DW, PBS contains inorganic salts, which have monovalent cations. Table I shows the composition of PBS and RPMI. No significant difference was shown between the mean value of PBS group and that of DW group. RPMI media contains Ca^{2+} and Mg^{2+} divalent ions, however, no significant difference was seen between the mean value of DW group and that of RPMI group. The mean value of RPMI group was lower than those of the other two groups, exhibiting that the physiologic concentration of divalent ions did not influence on hFN III 9-10 adsorption to titanium.

Many tissue cells need the formation of focal adhesions to grow and to differentiate.[20] RGD peptide is a cell-binding domain, which has a central role in the formation of focal adhesion.[21] hFNIII 9-10 shows similar adsorption patterns in affinity to titanium and was not influenced by surrounding ions in physiologic solution.

CONCLUSION

In our study, the adsorption the affinity of hFNIII9-10 to Ti and the effect of the surrounding ionic composition on the adsorption process were investigated. Compared to human fibronectin, hFNIII 9-10 contains RGD sequence and its synergic motif. hFNIII 9-10 showed the limit of adsorption amount and surrounding ions in physiologic solutions were not increase hFNIII 9-10 adsorption.

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