A TISSUE RESPONSE TO THE TITANIUM ALLOY (Ti-13Zr-6Nb) *IN VIVO*

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Statement of problem. Mechanisms of tissue-implant interaction and the effect of the implant surface on the behavior of cells has not yet been clarified.

Purpose. This study was performed to investigate the tissue reaction to the titanium alloy submerged into rat peritoneum *in vivo*.

Materials and methods. Titanium alloys (titanium-13Zirconium-6Niobium) were inserted inside the peritoneal cavity of Sprague Dawley rats. After 3 months, the tissue formed around the inserted titanium alloys were examined with a light-microscope. Tissue reaction around the material was analyzed by confocal microscopy to evaluate their biocompatibility in a living body.

Results. In *in vivo* study, foreign body type multinucleated giant cells were found in the fibrous tissue formed as a reaction to the foreign material (4 in 20 cases), but the inflammatory reaction was very weak. After experiment, the contaminants of biomaterials was removed from living tissue. In confocal microscopy, we observed that the staining of vinculin and actin showed mixed appearance. In a few cases, we found that the staining of vinculin and beta-catenin showed the prominent appearance.

Conclusion. We found that titanium-13Zirconium-6Niobium alloy was an excellent biomaterial.

Key Words

Titanium-13Zirconium-6Niobium alloy, Confocal microscopy, Biocompatibility

Titanium (Ti) is biologically compatible with bones, has high corrosion resistance, can be easily prepared in any required shape, and does not induce immunological reactions.[1] The interaction between the implant material and the surrounding tissues is believed to be one of the factors determining implant success.[2] The exact surface characteristics necessary for optimal osteointegraton are still not fully understood. The surface composition and structure influence the kinetics of protein adsorption and the structure of the adsorbed proteins.[3] Various implants which are inserted inside the body, are produced rapidly on a commercial scale for medical treatments. It is predicted that implants are not only to be used for

dental treatments, but also to insert computer chips into the skin, muscles, brain tissues, eyeball and many other organs. The corrosion resistance of the alloy and the toxicity of the individual metals that make up the alloy are the main factors determining biocompatibility.[4] Highly biocompatible pure titanium metal is the mostly recommended, but Ti-6A1-4V titanium alloys with higher strength are still in use. Biocompatibility of aluminium and vanadium are of a question in a living body. Therefore, Ti-13Zr-6Nb titanium has been suggested as an another solution. Zirconium (Zr) was substituted for aluminium (Al) and niobium (Nb) was substituted for vanadium (V) in Ti-13Zr-6Nb alloys. Using aluminium or vanadium containing alloys causes a concern since aluminium is a growth inhibitor of bone and a possible cause of Alzheimer' s disease, as well as vanadium has strong cytotoxicity.[5] Nb and Zr have also been studied and used as implant materials, and it has been reported that these metals possess good biocompatibility for biomaterials.[6] Mechanisms of tissue-implant interaction and the effect of the implant surface on the behavior of cells has not yet been clarified. The process by which cells become established on a surface involves initial attachment, followed by cellular spreading, secretion of extracellular matrix and cellular migration, cell growth and differentiation.[7] An understanding of the integrin subunits involved in osteoblast adhesion provides essential information for biomaterial improvement and notably for definition of the proteins which may be useful to adsorb on materials before implantation. In the cell cytoskeleton, actin appears pronounced when the cell is spread and becomes active but is not well pronounced when the cell has just attached.[8] Focal contacts can be identified by the presence of the actin binding protein, vinculin.[9] The purpose of the in vivo experiment is to place titanium alloys into the rat peritoneum and to investigate the tissue to the titanium alloys using a SEM and a confocal microscope.

MATERIALS AND METHODS

1) Animals

Healthy, with in the weight of 200 g to 250 g, without sex distinction, eight Sprague Dawley rats had a adaptation period to be used in an experiment.

2) Materials

Titanium alloys were fabricated by KIST(Seoul, Korea). Four types of coin-shaped titanium alloys that was in used in this experiment was titanium alloy which was 4 mm in diameter, 1 mm thickness coinshaped circle and 1mm diameter hole in the middle. The chief constituents of this titanium alloys was Ti, Zr and Nb. Observation of biomaterials surface by X-ray scanning analytical microscope. Table I and Fig. 1 showed the impurities and the physical properties.

3) Procedure

Ketalar 10-20 mg (ketamine HCL,Yuhan Co.) was injected into the peritoneum for a light general anesthesia. The prearranged part of the surgery was shaved and incision was made in abdominal region, and the disinfected coin shaped implant was inserted into the skin. The skin was suture with 3-0 silk and antibiotics was not used. During 12 weeks of the experiment all eight mice were healthy. Than those mice were sacrificed with an overdose of pentothal sodium (Abbot Laboratories Chicago, IL) to incise their skin and elevate, to extract the implant and it' s surrounding tissue. A saline solution was used to rinse out excessive blood and impurities and than was fixed in neutral formalin (10%) solvent. Tissue surrounding the implant was divide without damage and was embedded in paraffin. The tissues blocks

Table I. Atomic contents (%) of elements on titanium alloy surfaces before study

Specimens	Ti	Zr	Nb	Sn	C	O
sample 1	53.96	5.23	4.97	0.59	16.83	18.43
sample 2	51.30	4.77	4.67		17.70	21.56
sample 3	57.40	5.19	5.15		12.04	20.23
sample 4	69.56	6.40	6.24		17.81	

Atomic contents were measured by EDX

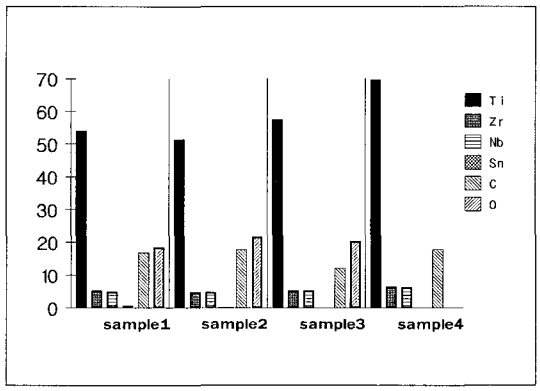

Fig. 1. The surface characterization of the titanium alloy samples by EDX (energy dispersive X-ray analysis, HORIBA 7200-H). Relatively small carbon and oxygen signals due to surface contaminations are observed in all samples.

were sectioned, on the surface side that' s being observed, to stained with hematoxylineosin (HE). These specimens were histopathologically observed with an light microscope.

4) Immunofluorescence analys is in rat tissues

Using immunofluorescence experiment, formalin-fixed paraffin sections of the peritoneal tissue were stained with antibodies to vinculin and beta-catenin, each at 1:50 in a block buffer. Briefly, 8-10 ㎛ thick tissue sections were deparaffinized and were then incubated with 5% blocking goat and mouse serum for 10 min with through intervening washes with PBS. The sections were incubated for 48 h at 4℃ with antibodies. After washing in PBS, the coverslips were rinsed three times with PBS and incubated for 1 h with goat anti-mouse FITC (Jackson Laboratories, West Grove, PA) and/or goat anti-mouse Texas-Red (Jackson Laboratories), each at 1:500 in a block buffer. They were again rinsed three times with PBS and mounted on glass slides using Fluoro GuardTM Antifade Reagent (Bio-Rad Laboratories, Hercules, CA). Confocal images were obtained from a MRC-1024 laser confocal microscopy (Bio-Rad).

RESULTS

1) Analysis of atomic contents of titanium alloy surfaces after study

Table II and Fig. 2 show changes of atomic concentration (%) of elements on titanium alloy. In four samples, oxygen contents was created from sample 4 and all other samples have lost carbon and oxygen contents.

2) Histomorphometric analysis

Table III shows summary of histologic findings on tissues around biomaterials. Among 32 cases, 20 cases were successfully examined while 12 cases could not be tested because of the bad quality of the samples such as separation between material and tissue. Fig. 3 shows thick fibrosis surrounding the biomaterials. In all the cases, a fibrous tissue layer which is being arranged and parallel to the surface biomaterials shows good biocompatibility. Fig. 4 shows microscopic finding of capillary proliferation. Many capillaries and

immature blood vessels were grown into the base of biomaterials lesion, so it is considered to induce a neoangiogenesis. Inflammatory cells (macrophages, plasma cells) were present in 4 cases of 20 cases. Also, the number of chronic inflammatory cells (lymphocytes) was not significantly increased in all the cases. In a few cases (4 in 20 cases), foreign body type multinucleated giant cell were found to the reaction of the foreign materials surrounded by fibrous tissue, but the inflammatory reaction was very mild. (Fig. 5)

3) Immunofluorescence analysis

Fig. 6 shows that the Focal contacts in tissue were visualized by vinculin staining. Cells grown on metallic surfaces also expressed well-developed round or elongated focal contacts at their peripheries. and focal contacts are seen as bright elongated at cell peripheries. Numerous multicellular islands are seen on biomaterials. As the spreading of attached cells, bundles of actin fibers forming stress fibers appeared in the cells on all materials. Fig. 7 shows that the merged appearance of vinculin

Table II. *Atomic contents (%) of elements on titanium alloy surfaces after study*

specimens	Ti	Zr	Nb	Sn	C	O
sample 1	82.92	8.37	7.89	0.87		
sample 2	83.98	7.79	8.23			
sample 3	83.27	8.43	8.30			
sample 4	53.35	5.12	5.03			36.50

Atomic contents were measured by EDX

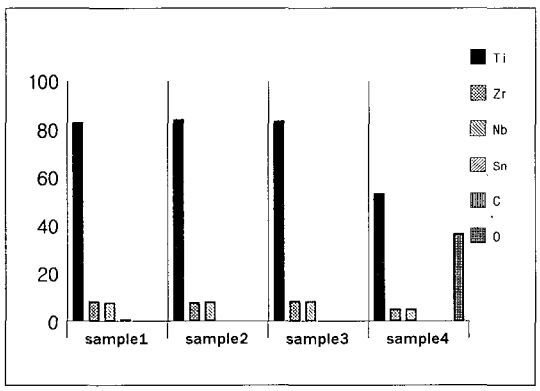

Fig. 2. The surface characterization of the titanium alloy samples by EDX (HORIBA 7200-H). It shows changes of atomic concentration (%) of elements on titanium alloy.

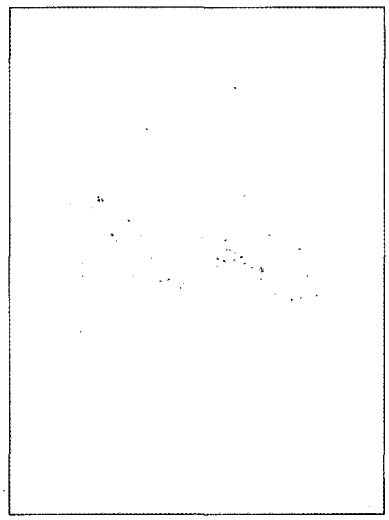

Fig. 3. Microphotograph showing thick fibrosis surrounding the biomaterials. In all the cases, fibrous tissue layer that' s being arranged and parallel to the surface biomaterials shows good biocompatibility (sample 4-a, HE, 100×).

Table III. Summary of histologic findings in every cases of biomaterials (+++ : marked, + : present)

	fibrosis	blood vessel	lymphocyte	macro-phage	multinucleated giant cell	plasma cell	mast cell	eosinophil	neutrophil
sample 1-a	+++	mild	moderate	mild	+			+	
sample 1-b	+++	severe	severe		+	+			
sample 1-c	+++	severe	mild	mild					+
sample 1-d	+++	severe	mild	mild		+			
sample 1-e	+++	severe	moderate	mild					
sample 1-f	+++	severe	few				+		
sample 1-g	+++	severe	few						
sample 1-h	+++	severe	few						
sample 2-a	+++	severe	mild						
sample 2-b	+++	severe	few						
sample 3-a	+++	severe	mild		+				
sample 3-b	+++	severe	few						
sample 3-c	+++	severe	few						
sample 3-d	+++	severe	few						
sample 4-a	+++	severe	mild						
sample 4-b	+++	severe	severe	severe				+	
sample 4-c	+++	severe	severe	severe		+			
sample 4-d	+++	severe	mild		+				
sample 4-e	+++	severe	mild			+	+	+	
sample 4-f	+++	severe		mild					

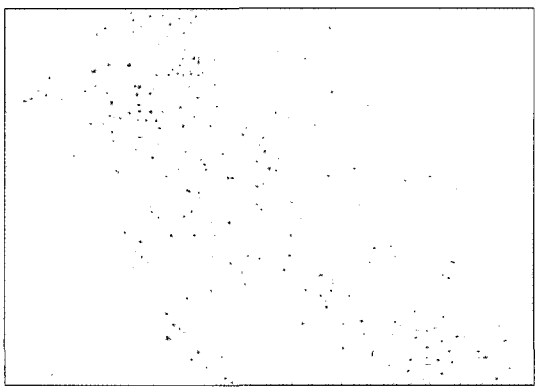

Fig. 4. Microscopic image showing capillary proliferation and moderate infiltratration of mononuclear inflammatory cells. Many capillaries and immature blood vessels growing into the base of biomaterials lesion are shown (sample 4-e, HE, 200×).

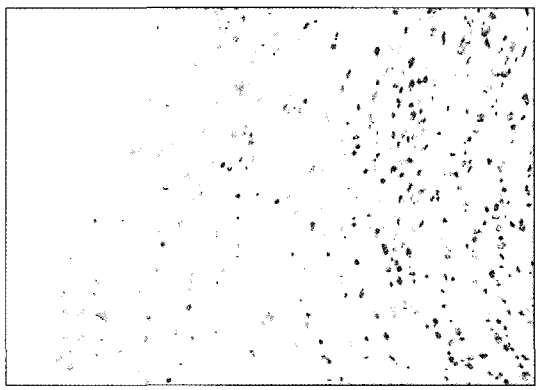

Fig. 5. High power view of foreign body type multinucleated giant cells accompanying chronic inflammation. In a few cases (4 in 20 cases), foreign body type multinucleated giant cells were found to the reaction of the foreign materials surrounded by fibrous tissue (sample 1-b, HE, 400×).

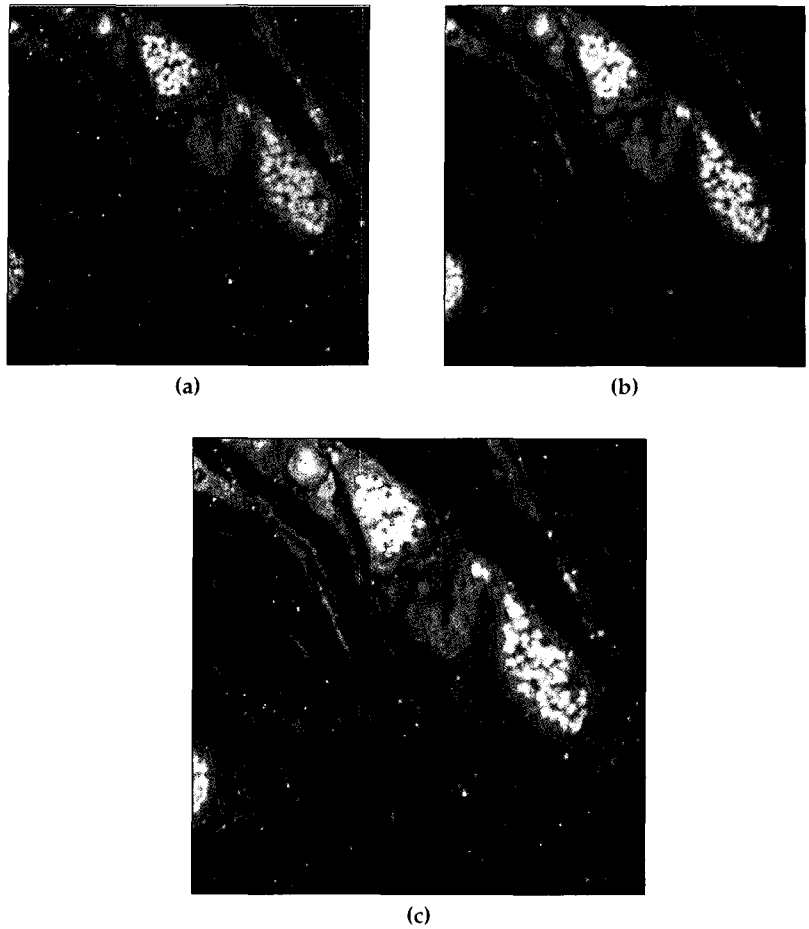

Fig. 6. Confocal microscopic images of tissue grown on biomaterials. Actin is shown in green and vinculin red. (a) actin staining of the sample 3-d (b) vinculin staining of the sample 3-d (c) merged appearance (400×).

and beta-catenin have prominent color.

Vinculin staining shows green color. Beta-catenin staining shows green color.

DISCUSSION

For the basic pathology research, a skin tissue is very useful part of the study and it' s most preferred part from the biomaterial evaluation. A poisonous reaction was reported from vanadium and aluminum in the Titanium alloys. An argument on biocompatibility of the biomaterial has been specialized to report that the process to become a mature osteoblast is impeded and the Titanium 13Zirconium 6Niobium alloys, a titanium alloys that' s highly adapted to living tissue was implanted inside the muscles and bone tissue and the result of both experiment was excellent. A general opinion on a divided tissue examination of the abdominal cavity came to a con-

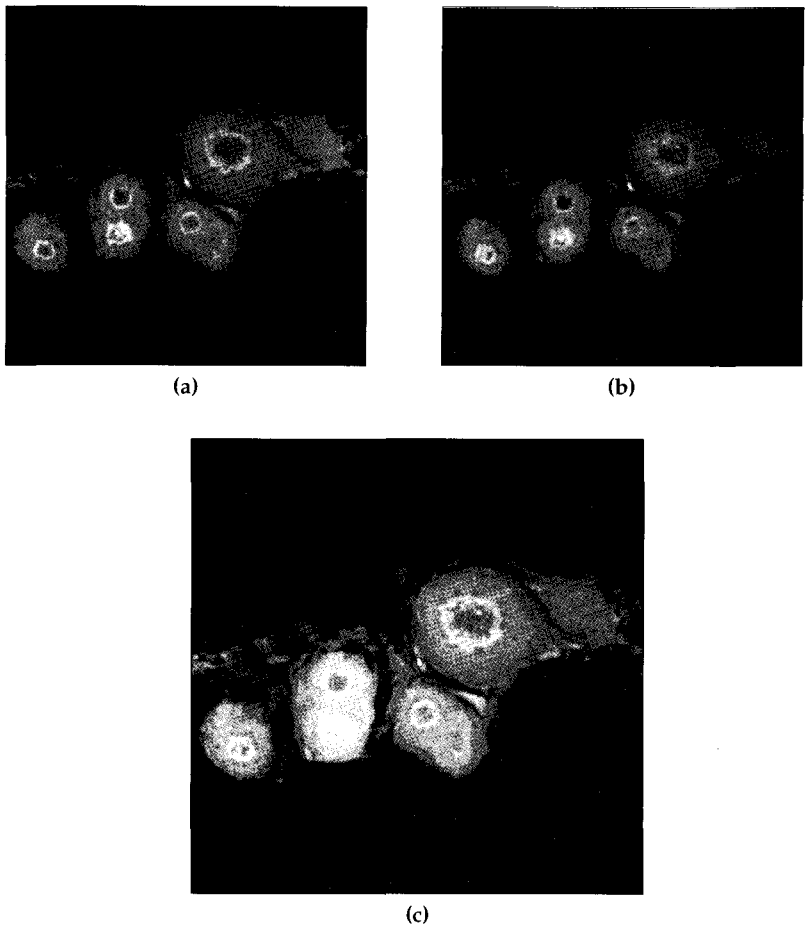

Fig. 7. Confocal microscopic images of tissue grown on biomaterials. Vinculin is shown in green and beta-catenin red. (a) vinculin staining of sample 1-7 (b) beta-catenin staining of sample 1-7 (c) merged appearance (400×).

clusion that the fibrotic wall has an slight inflammation like lymphocytes and neutrophils and hyalinization has occurred in a blood vessel. Foreign body Giant cell and fibrous tissue that' s found showed very mild. The oxide layer spontaneously formed on Ti surfaces appears to allow the adsorption of physiological fluids and proteins and the consequent adhesion of hard and soft tissues in a close contact to the surface. Surface purity is of major concern in periimplant healing and homeostasis. our EDX results showed that all surfaces were contaminated with hydrocarbon layers, which are typical of all implant materials used today. It is thus evident that the formation and maintenance of the periimplant epithelial seal is governed by a variety of physicochemical surface properties.[10]

The implant, in the biological fluid environment of the implant site, adsorbs a layer of proteins. This process, really a surface modification by proteins, takes seconds and is observed with essentially all materials. Shortly after protein

adsorption, neutrophils interrogate the implant (really the adsorbed proteins at the surface of the implant). Unless bacteria or endotoxin (from bacterial cell walls) are found, the neutrophils numbers will diminish at the implant. However, by about one day, macromaphages will be seen to accumulate at the implant. The macrophages will attempt to engulf and digest the implant as a foreign to engulf and digest the implant as a foreign body. They will, of course. be unsuccessful and, apparently, in an attempted to enhance their effectiveness in the engulfment process, they will fuse to form giant cells. In a process often called frustrated phagocytosis, the giant cells will send chemical signals bringing fibroblasts to the implant site (typically at one week+) The fibroblasts will encapsulate the implant in a thin, avascular collagenous bag to isolate it from the body. This process is often called the foreign body reaction. For a "biocompatible" implant, the reaction site after three to four weeks will be relatively quiescent. However, at the interface between the capsule and the implant, mildly activated macrophages and giant cells will be observed, even years after the implantation.[11] In our experiment, in a few cases, foreign body type multinucleated giant cell were founded to the reaction of foreign materials surrounded by fibrous tissue. It is very mild inflammation. Glowacki et al. demonstrated that macrophage and giant cells in the process of resorption were involved in implant degradation.[12] Macrophages secrete a large number of different factors, including cytokines and growth factors[13] at the contact with material surfaces.[14-16] Cytokines (including interleukin-1) have been shown to induce bone resorption.[17] and fibroblast proliferation. However, the nature of the multinucleated cells elicited in contact with biomaterials is still uncertain.[12,18,19] In immunofluorescence assay, it showed that vinculin and actin have mixed appearance, but showed that vinculin and beta-catenin have prominent color (red and green). In tissue, it was difficult to examine those protein. we just tried to adopt confocal microscopy in tissue field. Although various materials have been used as dental materials for a longtime, factors and mechanisms underlying the biological response to them are poorly understood. Further studies are needed to clarify how the transmission of signals in osteoblastic cells on biomaterials affects the formation of bone matrix and how implant surface roughness affects osteoblast gene expression.

CONCLUSION

1. We found that Titanium-13Zirconium-6Niobium alloy was an excellent biomaterial.
2. After experiment, We found that the contaminants of biomaterials was removed from living tissue.
3. In confocal microscopy, we observed that the staining of vinculin and actin showed mixed appearance. In a few cases, we found that the staining of vinculin and beta-catenin showed the prominent appearance.

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