In vitro Study of Collagen Coating of Titanium Implants for Initial Cell Attachment

Megumi NAGAI, Tohru HAYAKAWA, Akira FUKATSU, Masafumi YAMAMOTO, Masahiko FUKUMOTO, Fumio NAGAHAMA, Hiroyuki MISHIMA, Masao YOSHINARI, Kimiya NEMOTO, and Takao KATO

Department of Comprehensive Clinical Dentistry, Research Institute of Oral Science
1 Department of Dental Materials, Research Institute of Oral Science
2 Department of Oral Medicine, Research Institute of Oral Science
3 Department of Anatomy, Research Institute of Oral Science,
Nihon University School of Dentistry at Matsudo
2-870-1, Sakaecho-nishi, Matsudo, Chiba 271-8587, Japan
4 Department of Dental Materials Science and Oral Health Science Center, Tokyo Dental College,
1-2-2 Masago, Mihamaku, Chiba 261-8502, Japan

Received April 3, 2002/Accepted June 24, 2002

The aim of this study was to examine the influence of collagen coating on titanium on the initial attachment of human gingival fibroblasts for the development of the implant with peri-implant soft tissue attachment. The morphological changes of cultured human gingival fibroblasts were investigated by scanning electron microscopy (SEM). Four different surfaces, i.e. non-coated mirror-polished titanium, collagen-coated titanium, non-coated tissue-culture polystyrene, and collagen-coated polystyrene were examined. Collagen coating of titanium was effective for enhancing the initial cell attachment. It is expected that collagen coating of titanium implants will improve the attachment of the peri-implant soft tissue to titanium at early stages after the implantation. SEM observation revealed the morphological effect of collagen coating on both titanium and polystyrene surfaces. Many lamellipodia and filopodia were recognized on collagen-coated titanium or polystyrene. Collagen coating improved the activity of human gingival fibroblasts.

Key words: Titanium implant, Collagen, Human gingival fibroblast

INTRODUCTION

Presently titanium and titanium alloy implants are widely used as biomaterials for dental implants because of their superior mechanical properties and their direct bone anchorage called osseointegration. The direct bone contact of titanium and titanium alloy implants produce suitable stability into the bone tissue. However, implant failures sometimes occur due to inflammatory gingival breakdown around the implant material. This is caused by the accumulation of bacterial plaque surround-
ing the implant surface.

There is a basement membrane between human natural teeth and the cell membrane. The cells adhere to this base membrane by means of hemidesmosomes. Jansen et al.\(^5\) reported that hemidesmosomal contacts were found only on apatite and polystyrene implants and not on various metals including titanium implants. A recent ultrastructural study revealed that hemidesmosomes were rare and absent in the upper and middle regions of the peri-implant epithelium-implant interface, and existed only in the lower region\(^6\). These studies supported the poor attachment of peri-epithelium soft tissues to the implant materials. This poor attachment caused the bacteria invasion around the titanium implant.

There have been many studies on the chemical and physical modifications of titanium implant materials to obtain stable and tight contact to bony tissues, for example, calcium phosphate coating\(^7,8\) or greater surface roughness\(^9,10\). However, few studies related to the improvement of the gingival tissue attachment to the implant materials have been reported. The first biological reaction at the biomaterial-tissue interface is the adsorption of body fluid proteins including extracellular matrix components onto its surface after the implantation of biomaterials into a foreign body\(^11-13\). It is widely accepted that these adsorbed proteins control the subsequent biological response of the implant-tissue interface\(^14,15\). Coating of the implant surface with cell-adhesive proteins will improve the cell activity at the implant-tissue interface, and will improve the connection between the implant and peri-implant soft tissue.

Some studies for the coating of the titanium surface with cell-adhesive proteins have been reported. Dean et al.\(^16\) investigated the effectiveness of the coating of titanium with fibronectin or laminin on the attachment of human gingival cells or human epithelial cells. They found that fibronectin coating of titanium implants enhanced the gingival fibroblast binding and laminin coating of titanium implants enhanced the gingival epithelial cell binding. Cannas et al.\(^17\) reported that the coating of titanium with fibronectin enhanced the adhesive characteristics of baby hamster kidney fibroblasts. Endo\(^18\) evaluated the efficacy of immobilized fibronectin on the human gingival cell culture. It was also reported that the coating of titanium with collagen exhibited favorable effects on the initial adhesion and growth activities of rat calvarial osteoblasts\(^19,20\). Morikawa et al.\(^21\) reported the immobilization of collagen onto the titanium surface using gold deposition and production of a monomolecular layer with cystein.

There have been few studies related to the collagen coating of titanium substrate on human gingival cell activity. In the present, we examined the initial attachment of human gingival cells by the collagen coating on the titanium surface for the development of the implant with peri-implant soft tissue attachment. The morphological influence of human gingival cells by the collagen coating was also evaluated by scanning electron microscopy (SEM).
MATERIALS AND METHODS

Titanium substrate

Commercially pure wrought titanium disks (JIS, Japan industrial Specification H4600, 99.9 mass% Ti, Furuuchi Chemical Corp., Tokyo) with a diameter of 15 mm were used. They were ground down to 1200 grit, finally polished using colloidal silica with pH 9.8, and then ultrasonically cleaned with acetone. Before use in the cell culture experiments, all polished titanium disks were autoclaved for 30 min at 120°C.

Cell isolation and cultures

Cells used in in vitro experiments were from pieces of gingival tissue by the excision during routine periodontal surgical procedures at the Dental Hospitals attached to Nihon University School of Dentistry at Matsudo. The study was explained to the patients and their informed consent was obtained. Small pieces of tissue were washed several times with Dulbecco’s Modified Eagle Medium (DMEM, Gibco BRL, Rockville, MD, USA) containing 100 U/ml penicillin G (Gibco BRL, Rockville, MD, USA), 100 μg/ml streptomycin (Gibco BRL, Rockville, MD, USA), 0.2 μg/ml amphotericin B (Gibco BRL, Rockville, MD, USA). The epithelial layer was then mechanically separated from the connective tissue with sterile forceps. Primary cultures were incubated in an atmosphere of 5% CO₂, 95% air at 37°C in the following medium, DMEM supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin G, and 0.2 μg/ml amphotericin B, and routinely passaged using 0.25% trypsin and 0.02% EDTA in Dulbecco’s phosphate-buffered saline. The cells were fed twice per week and subcultured using 0.25% trypsin. Cells for use in the experiments described were used between passages 15-20. Phase contrast micrography of primary human gingival fibroblasts are shown in Fig. 1.

![Fig. 1 Phase contrast micrograph of primary cultured human gingival fibroblasts. (original magnification × 40)](image-url)
Collagen coating
Collagen type I of commercial pig derivation (Nitta Gelatine, Osaka, Japan) (3.0 mg/ml, pH 3.0) was dissolved into distilled water, and was diluted ten times to adjust to 300 μg/ml according to the manufacturer’s instruction.

For coating with collagen, titanium disks were placed in a 24-well culture dishes (FALCON, Becton Dicknson Labware, NJ, USA), and were exposed to a 0.5 ml collagen solution at room temperature for 1 h. Then, they were rinsed with phosphate buffer saline (PBS, pH=7.4) solution twice. Tissue-culture polystyrene dishes were also coated with collagen as the same method described above. The presence of coated collagen was identified by X-ray photoelectron spectroscopy (ESCA-750, Shimadzu, Kyoto, Japan) measurement. Before collagen coating, no peak of 1s electron of nitrogen (N1s) was detected on titanium and polystyrene. A N1s peak from coated collagen was observed at about 402 eV of binding energy as shown in Fig. 2.

Thus, we examined four different surfaces, i.e. non-coated tissue-culture polystyrene, collagen-coated polystyrene, non-coated mirror-polished titanium, and collagen-coated titanium.

Cell attachment assay
When the outgrown cells became confluent after 15-20 passages, they were treated with a trypsin solution, and resuspended in DMEM. The suspension was plated on the non-coated or collagen-coated titanium disks in 24-well culture dishes at a cell population density of 1×10^6 cells/ml. The cell suspension was also plated in non-coated tissue-culture polystyrene and collagen-coated polystyrene. The cell suspension was allowed to incubate for 1.5 hr under standard culture conditions. Afterwards, cells were washed three times with PBS, and non-adherent cells were
washed away. The adherent cells were removed from the titanium disks or polystyrene by trypsinization and the number of adherent cells were counted using a Coulter counter (Nikkaki, Tokyo, Japan). The viable cells in a hemocytometer was counted using the trypan blue dye exclusion assay, and the cell viability was determined from the ratio of the number of viable cells towards the number of initial attached cells. Unstained cells were counted as viable cells. Cell attachment experiments were performed in triplicate. The data were analyzed by t-test at p=0.05.

Scanning electron microscopy (SEM) observation
After incubation of the test materials as described above, culture wells containing test materials were rinsed three times with PBS. Attached cells were fixed with 1% glutaraldehyde for 15 minutes, rinsed three times with PBS, and then fixed with 2% osmium tetroxide for 15 minutes. Fixed cells were taken in stages of 5 minutes each (50%, 70%, 80%, 90%, 95%, 99.9%) to 100% ethanol, dried using critical drying apparatus and ion-coated with gold and palladium. The morphology of cells was observed with a scanning electron microscope (S-2150, Hitachi, Tokyo, Japan).

RESULTS

Fig. 3 shows the number of adherent cells on non-coated or collagen-coated tissue-culture polystyrene and non-coated or collagen-coated titanium. The number of adherent cells on collagen-coated polystyrene was approximately 570 cells/cm² which is identical to that seen in non-coated polystyrene plates. On the other hand, coating of collagen on the titanium surface resulted in a significant increase of adherent cells. The number of adherent cells on non-coated titanium was approximately 450 cells/cm², and that on collagen-coated titanium was approximately 930 cells/cm². The number of attached cells increased almost two-fold by collagen coating.

The cell viability obtained from trypan blue staining is shown in Fig. 4. The cell
viability was approximately 70% on the different surfaces, i.e. non-coated or collagen-coated polystyrene and non-coated or collagen-coated titanium surfaces, and there were no significant differences among each surface (P>0.05).

SEM pictures of human gingival fibroblasts adhered to different materials are shown in Figs. 5-8. Figs. 5 and 6 show the SEM pictures of attached cells on non-coated and collagen-coated tissue-culture polystyrene, respectively. The SEM pictures revealed the differences in the surface appearances and shapes of attached cells between non-coating and collagen-coated polystyrene, although there was no significant difference among the number of adherent cells. The attached cells on non-coated polystyrene showed a roughened surface appearance, and showed a rounded shape and slight formation of filopodia (thin and stiff protrusions, Fig. 5a, b). The cells attached on collagen-coated polystyrene showed smooth surface appearance and a

![Fig. 5 SEM pictures of human gingival fibroblasts on non-coated tissue-culture polystyrene.](image1)
a) lower magnification (bar=200μm), b) higher magnification (bar=20μm)

![Fig. 6 SEM pictures of human gingival fibroblasts on collagen-coated tissue-culture polystyrene.](image2)
a) lower magnification (bar=200μm), b) higher magnification (bar=20μm)
more flattened and spread shape. They exhibited more spanning of filopodia and lamellipodia (thin and sheet-like leading edge) as shown in Fig. 6a, b.

Figs. 7 and 8 show the SEM pictures of attached cells on non-coating and collagen coating titanium surface, respectively. The cells attached on non-coated titanium showed the rounded shape and roughened surface appearance. No filopodia nor lamellipodia existed (Fig. 7a, b). On the other hand, cells attached on the collagen-coated titanium surface showed a flattened and spread shape, and smooth surface appearance. The presence of lamellipodia and filopodia was confirmed (Fig. 8a, b).

**DISCUSSION**

In this study, we evaluated the efficacy of collagen coating of titanium or polystyrene on human fibroblast activity.
Collagen is a well-known cell-adhesive protein and is effective in promoting cellular adhesion and spreading. Collagen possesses RGD (arginine-glycine-aspartic acid) sequences, which are recognized by integrins such as fibronectin or laminin. It is predicted that the collagen coating of titanium implants will improve the attachment of peri-implant soft tissue to titanium implant materials.

In the present study, human gingival fibroblasts obtained from the primary culture were used to evaluate the effect of collagen coating of titanium on peri-implant soft tissue adhesion. Human gingival fibroblasts actually adhere to the implant surface when the implant is inserted into the tissue. However, there have been few studies related to human gingival fibroblasts assay. It is possible to obtain some useful information for the improvement of the poor attachment of peri-epithelium soft tissues to the implant surface from the results of human gingival fibroblasts assay.

The adhesion of fibroblasts on titanium is a prerequisite for soft tissue integration. In the present study, the number of adherent cells on collagen-coated titanium was significantly greater than that on non-coated titanium. The previous studies of osteoblasts reported that collagen coating of titanium accelerated the initial adhesion of osteoblasts. The findings of the present study suggested that collagen coating of titanium was effective not only for osteoblast but also for human gingival fibroblasts in the enhancement of the initial cell attachment.

Cell viability of human gingival cells was also determined. The higher cell viability means less cytotoxicity. The viability of cells on non-coated and collagen-coated titanium or polystyrene was approximately 70%, although the number of adherent cells was different. These results suggested that both titanium and polystyrene possessed equal properties in terms of cytotoxicity with or without collagen coating, and that four different surfaces employed in the present study caused no severe cytotoxicity.

The number of adherent fibroblasts was determined after 90-min cell culture. In the clinical situation, it is better to acquire the strong adhesion of peri-implant soft tissue to implant materials as soon as possible. The present study revealed the collagen coating of titanium enhanced the initial attachment of human gingival fibroblasts within 90 min of cell culture. Thus, it is expected that collagen coating of titanium implants will improve the attachment of the peri-implant soft tissue to titanium at an early stage after the implantation. Further studies related to the time for cell culture are needed to clarify the effect of collagen coating on the time after implantation.

There were no effects on the collagen coating of tissue-culture polystyrene toward the initial cell attachment. It is widely accepted that the cells well attached on the hydrophobic surface. Human gingival fibroblasts attached to tissue-culture polystyrene. Thus, the effect of collagen coating on the number of attached cells was not clearly identified.

However, collagen coating markedly influenced the shape of the attached cells not only on titanium but also on polystyrene. On the collagen coated titanium or the polystyrene surface, a flattened and spread shape of cells was observed and the
presence of lamellipodia and filopodia were clearly recognized. The cells on non-coated titanium or polystyrene showed round shapes, and fewer lamellipodia and filopodia. This suggests that attached cells on collagen-coated titanium or polystyrene exhibited the higher migration ability on non-coated titanium or polystyrene. The improvement in the adherent ability of cells by collagen coating also produce a flattened and spread shape of the attached cells. It revealed that collagen coating was effective for the improvement of the cell activity by SEM observations, although the detailed cell activity such as actin fiber alignment should be investigated by immunostaining techniques.

In the present study, collagen was physically attached to the titanium disk, not chemically attached. The interface between collagen and the substrate, polystyrene or titanium, was not clear. The interface substance has a possibility to influence the results of the cell culture assay. Williams et al. investigated the adsorption and desorption behavior of albumin and fibrinogen on 17 different metal surfaces, and found that \textit{in vivo} substantial desorption of preadsorbed proteins occurred within 106 hours for most metals. Hayakawa et al. developed a simple immobilization technique of surface-adhesive protein on titanium. The influence of the immobilization of collagen on the titanium surface will be studied in the near future.

Periodontal fibroblasts will also influence the attachment of implants to peri-implant soft tissue. The influence of collagen coating on the periodontal fibroblast cell assay should be further investigated.

Finally, collagen coating of titanium enhanced the initial attachment of human gingival fibroblasts and improved the migration activity. Collagen coating of titanium has a possibility to develop a tight attachment of dental implants to peri-implant soft tissue.

CONCLUSIONS

Human gingival fibroblasts were cultured on four different surfaces, \textit{i.e.} non-coated mirror-polished titanium, collagen-coated titanium, non-coated tissue-culture polystyrene, collagen-coated tissue-culture polystyrene. Collagen coating was effective for enhancing the initial cell attachment on the titanium surface but not on the polystyrene surface by measuring the number of attached cells. The morphological effect by collagen coating was clearly recognized. Many lamellipodia and filopodia were recognized on collagen-coated titanium or polystyrene. It was revealed that collagen coating improved the activity of human gingival fibroblasts.

ACKNOWLEDGMENT

This study was supported by a Grant-in-aid for Research for Frontier Science and a Grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan to promote 2001-Multidisciplinary Research Projects (in 2001-2005).
REFERENCES


