

ORIGINAL ARTICLE

Rat Endothelial Cell Attachment, Behavior and Gene Expression on NaOH-treated Titanium Surfaces

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SYNOPSIS

The aim of this study was to evaluate the influence of nanostructures on the surface of titanium, titanium nanosheets (TNS), on the initial attachment, proliferation, and gene expression of various functional factors by rat aortic endothelial cells (RAEC) involved in wound healing. We found that the initial attachment and proliferation of RAECs after 15, 30, 45, 60, and 120 min of culture were increased on titanium surfaces modified with TNS. We also found that the expression of intercellular adhesion molecule 1, von Willebrand, and thrombomodulin transcription factors were elevated in samples containing a TNS-modified disk in compared with unmodified titanium disks during the early stages of wound healing. Our results suggest that nanostructures on titanium surfaces stimulate RAECs to upregulate expression of angiogenic factors and adhesion molecule genes, which play an essential role in controlling inflammation and revascularization during wound healing after implantation.

Key words: aortic endothelial cells, titanium, nanostructures, wound healing

INTRODUCTION

Materials scientists and clinicians worldwide are working to improve the performance of dental implants, with the aim of accelerating and maintaining integration into hard and soft tissues, and extending their range of application. The surface characteristics of the implant material affect the rate and extent of osseointegration¹. Vandrovцова *et al.*² recently reviewed the growing evidence

that surface-modified materials can increase adhesion, growth, and osteogenic differentiation of cells, thus promoting the integration of the implant into bone and maintaining its secondary stability.

Recent advances in dental implant research allow for the modification of the surface of the implant material at the nanometer scale^{3,4}. Surface modifications that increase surface area and

create a more fine surface roughness may yield better tissue-titanium mechanical interlocking⁵. More importantly, such nanoscopic features may also directly affect osteogenic cell behavior near implant fixtures through biomimicry; the modified alloplastic surfaces better replicate the natural cellular environment at the nanometer level^{3, 4, 6}. The titanium nanosheet structures (TNS) used in this study are nanostructures similar to the TiO₂ nanotubes created by titanium deposition using the process of TiO₂ sputtering⁷. Recent work has shown that nanotubes and TNS-modified can be created on titanium metal surfaces using treatment with 10 M NaOH aqueous solution at 30°C⁸, which we employed to create TNS-modified on modified titanium disks. Treatment with a NaOH aqueous solution produces a rough, nanoscale surface^{9, 10}, and scanning electron microscopy (SEM) images of our TNS-modified disks demonstrated consistent roughness, without any cracks.

The success of dental implantation depends initially on appropriate wound healing of both hard and soft tissues. The importance of endothelial cells (ECs) in bone formation around the implant material has been recognized in recent years. Several cells that play a role in bone formation are derived from the endothelium of the capillary system. Moreover, ECs play an important role in recruiting cells to the site of inflammation, chemotaxis, cell adhesion, and extravasation^{11, 12}. Implants are essentially installed into blood clots, which are formed by fibrin that is degraded in a complex sequence during fibrinolysis. The vascular endothelium is a focal point for the interactions of the coagulation and fibrinolytic enzyme pathways¹³. ECs play a key role in the fibrinolytic pathway through several direct and indirect mechanisms¹³. Angiogenesis is of primary importance during the initial

phase of the healing process. Characterizing the responses of cells involved in angiogenesis and bone formation adjacent to the implant is critical to understand promote implant biocompatibility and improve implant stability. If TNS-modified can favorably alter the cellular responses of ECs towards osseointegration, they could have a large impact on tissue regeneration and wound healing therapies. The aim of this study was to evaluate the influence of TNS-modified on titanium surfaces on the initial attachment, proliferation, and gene expression of various functional factors of ECs involved in wound healing.

MATERIALS AND METHODS

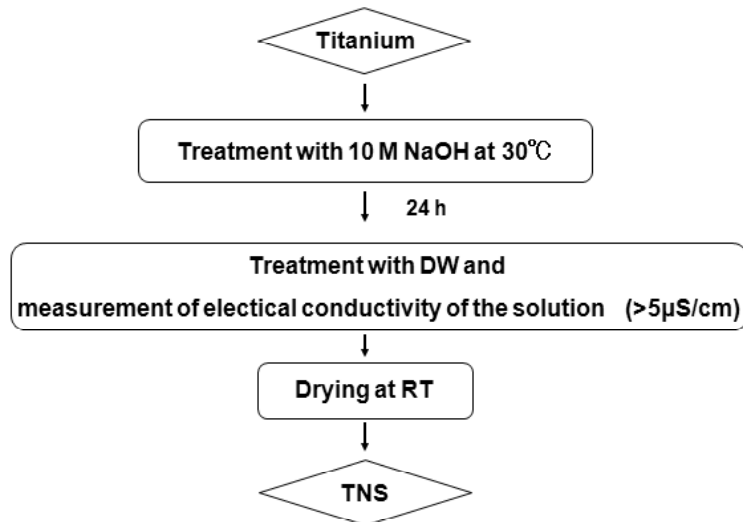
1. Specimen production

Table 1 shows the process of TNS fabrication. Titanium disks (15 mm diameter) were punched from 1 mm thick grade 2 unalloyed titanium sheets (Daido Steel, Osaka, Japan). These disks were immersed in 10 M NaOH (aq) and placed in an oil bath maintained at 30°C for 24 h; unprocessed titanium disks were used as controls with polishing by using sand paper before No.2000. The NaOH solution in each flask was replaced with distilled water (200 mL), and the NaOH treatment and wash procedure was repeated until the wash solution reached a conductivity of 5 µS/cm. Specimens were then dried at room temperature. All titanium disks were sterilized by dry heat sterilizer.

2. Cell culture

Rat aortic endothelial cells (RAEC) were isolated from the aorta of 8-week-old Sprague Dawley rats. Rats were euthanized after anesthesia with sodium pentobarbital intraperitoneally, and the thoracic aorta, from distal to the aortic arch to the diaphragmatic level, was dissected, removed, and carefully

Table 1 TNS deposition process



cleaned of connective tissue. RAECs were cultured in endothelium growth medium (HuMedia-MvG, Kurabou, Osaka, Japan) supplemented with 5% fetal bovine serum (FBS; Invitrogen, Life Technologies Corp., Carlsbad, CA, USA), 10 ng/mL recombinant human epithelial growth factor (Kurabou), 1 µg/mL hydrocortisone hemisuccinate (Kurabou), 50 µg/mL gentamicin (Kurabou), 5 ng/mL amphotericin B (Kurabou), 5 ng/mL recombinant basic human fibroblastic growth factor (Kurabou), 10 µg/mL heparin (Kurabou), and 39.3 µg/mL dibutyryl-cAMP (Kurabou) in 75 cm² culture flasks coated with type I collagen (Asahi Technoglass Inc., Tokyo, Japan). Cells were washed three times with washing buffer, mounted with CD31 reagent, and photographed using a microscope equipped with a digital camera to characterize the RAEC morphology. The cells were cultured at 37°C in a humidified 5% CO₂/95% air atmosphere. The media was changed every 3 days, and harvested cells were seeded and grown until reaching subconfluence. The cells from passages 4–6 were used in the following experiments. The cells were detached from the flasks by

trypsinization, washed twice in phosphate buffered saline (PBS), resuspended in culture medium, and seeded into 24-well tissue culture plates (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) containing test and control titanium disks. This study protocol was performed following the Guidelines for Animal Experimentation at Osaka Dental University (Approval No. 13-02038).

3. *In vitro* tube formation assay

In order to assess the biological activity of RAECs on TNS-modified titanium surfaces, we observed the surfaces' ability to stimulate angiogenesis *in vitro* using a three-dimensional endothelial culture. Tube formation was measured using an *In vitro* angiogenesis assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). First, the TNS-modified titanium surfaces in each well of 24-well tissue culture plates (Falcon) were coated with 100 µL of Cell-Based Extracellular Matrix Gel (Cayman Chemical Company). The Chamber slide was then gently shaken for 1 min to ensure that the gel was even. The coated titanium surfaces were next incubated at 37°C for 60 minutes to allow the gel to

solidify. A suspension of cells (100 μ L per well at 1×10^5 cells/mL) was seeded onto each gel-coated titanium surface. After 6 days of culture, the samples were stained with a calcein solution, and cell network structures were examined under an inverted fluorescence microscope (KEYENCE, Osaka, Japan).

4. Cell proliferation/viability assay

RAECs were harvested as above and seeded at a density of 4×10^4 cells/well into 24-well microplates containing test and control titanium disks. After rinsing with PBS, cell proliferation/viability were determined using the CellTiter-Blue™ Cell Viability Assay (Promega, Madison, WI, USA), an MTS assay after 15, 30, 45, 60, 120, and 180 min of incubation, according to the manufacturer's protocol. Briefly, following aspiration of supernatant, 100 μ L of CellTiter-Blue™ reagent diluted 6-fold in PBS was added to each well and incubated for 1 hour at 37°C. We measured fluorescence intensity (excitation: 560 nm, emission: 590 nm) using a multi-mode microtiter reader (SpectraMax M5, Molecular Device Inc, Sunnyvale, CA, USA).

5. Cell morphology

Confocal laser scanning microscopy was used to examine cell morphology and cytoskeletal arrangement in RAECs seeded onto the test and control titanium surfaces. After 15, 30, 45, 60, 120, and 180 min of culture, cells were fixed in 10% formalin and stained with the red fluorescent dye rhodamine phalloidin (actin filaments; Molecular Probes, Eugene, OR, USA). We examined the cell morphology on the test and control titanium disks under an inverted fluorescence microscope (KEYENCE).

6. Real-time PCR analysis

After 2 and 5 days of culture, total RNA was extracted from the cells and cDNA was synthesized from 1 μ g of RNA using

a High Capacity cDNA Archive Kit (Applied Biosystems Inc Foster City, CA, USA). Intercellular adhesion molecule 1 (ICAM-1) mRNA (after 2 and 5 days of culture), and von Willebrand factor and thrombomodulin mRNA expression (after 5 days of culture) were investigated by real-time reverse transcription polymerase chain reaction (RT-PCR) using a StepOne Plus™ Real-Time RT-PCR System (Applied Biosystems). In a Fast 96-well Reaction Plate (100 μ L total well volume; Applied Biosystems), 10 μ L of Taqman Fast Universal PCR Master Mix, 1 μ L of the Runx2 primer (TaqMan® Gene Expression Assays) 2 μ L of sample cDNA, and 7 μ L of DEPC water (Nippongene, Tokyo, Japan) were added to each well. The plate was subjected to 40 reaction cycles of 95°C for 1 s and 60°C for 20 s. The relative gene expression rates were calculated employing the $\Delta\Delta$ Ct method¹⁴ in each group normalized to the gene expression rate of the negative control group.

7. Statistical analysis

All experiments were performed in triplicate. Data are presented as mean \pm standard deviation. In all analyses, statistical significance was determined by Student's t-test, $P < 0.05$.

RESULTS

1. Characterization of rat endothelial cells

Immunofluorescence staining for CD31 produced a characteristic marginal staining pattern in the harvested endothelial cells.

2. In vitro tube formation assay

As showed in Fig. 1, RAEC spheroids were formed in the presence of TNS-modified, and capillary sprouts grew radially on TNS-modified and unprocessed titanium surfaces after 6 days of culture.

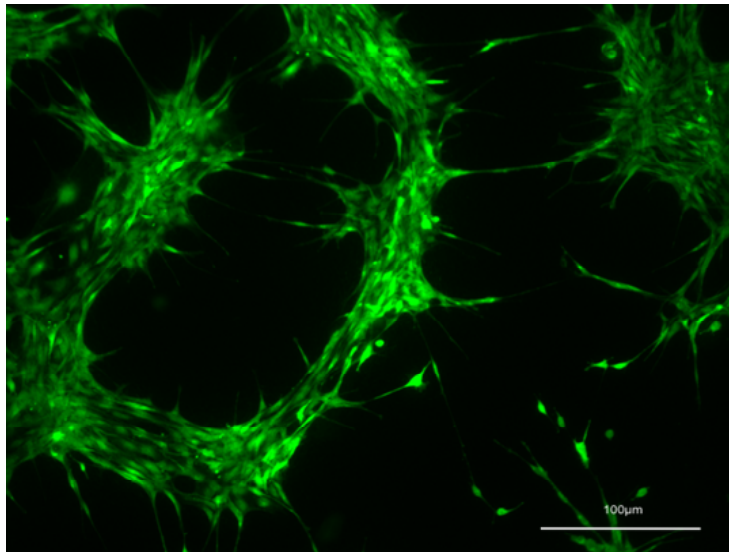


Fig. 1 The effect of TNS on *in vitro* tube formation was measured using a three-dimensional endothelial cell cultivation system (*In Vitro* Angiogenesis Assay Kit). RAEC spheroids were stimulated to form in the presence of TNS, and capillary sprouts grew radially on both TNS-modified and unprocessed titanium surfaces after 6 days of culture.

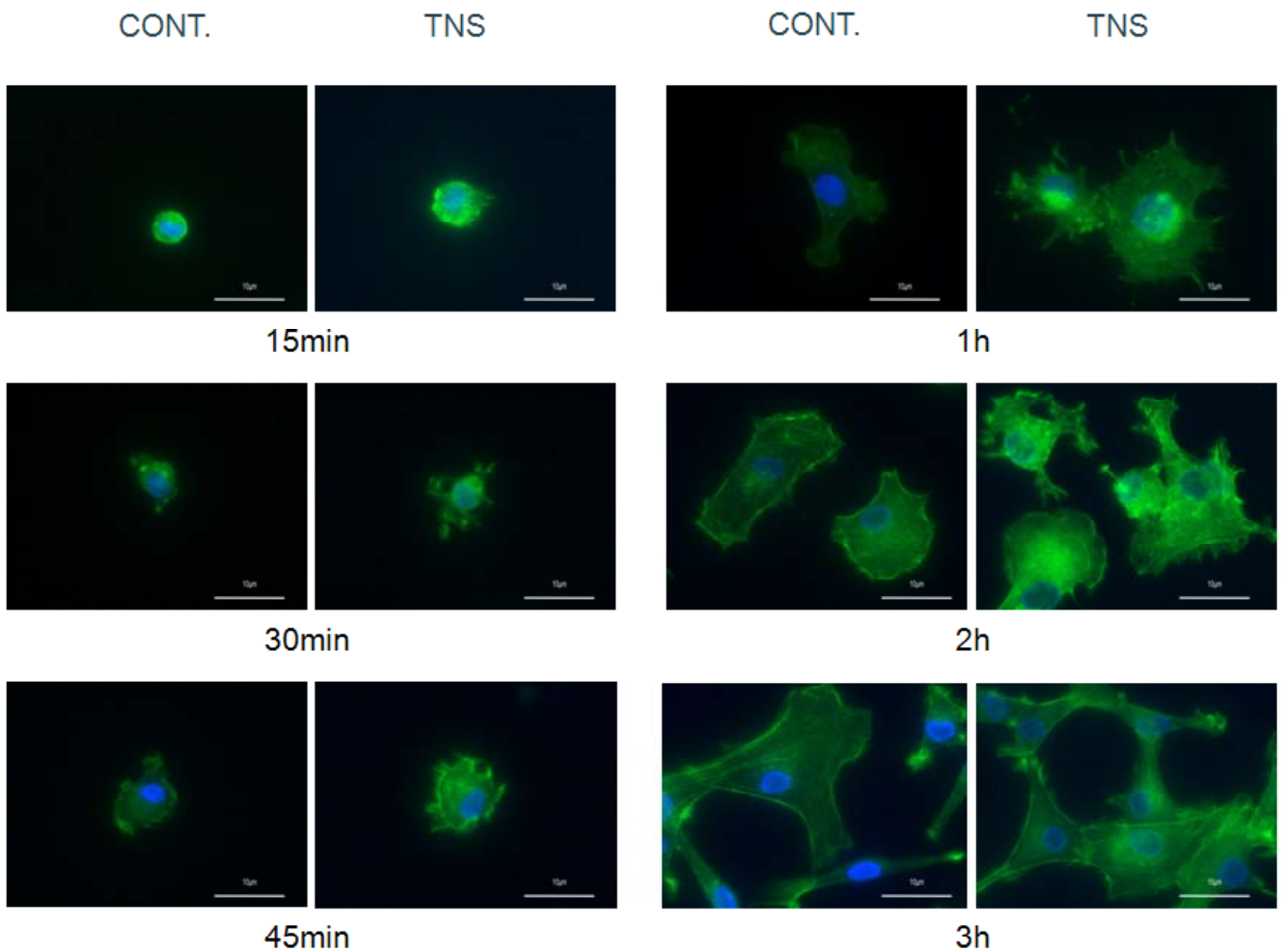


Fig. 3 Fluorescence images of the test and control groups. Images revealed flat cell adhesion in the control groups and extended adhesion of cell outgrowth in the test group. This tendency was clearly observed to progress with time.

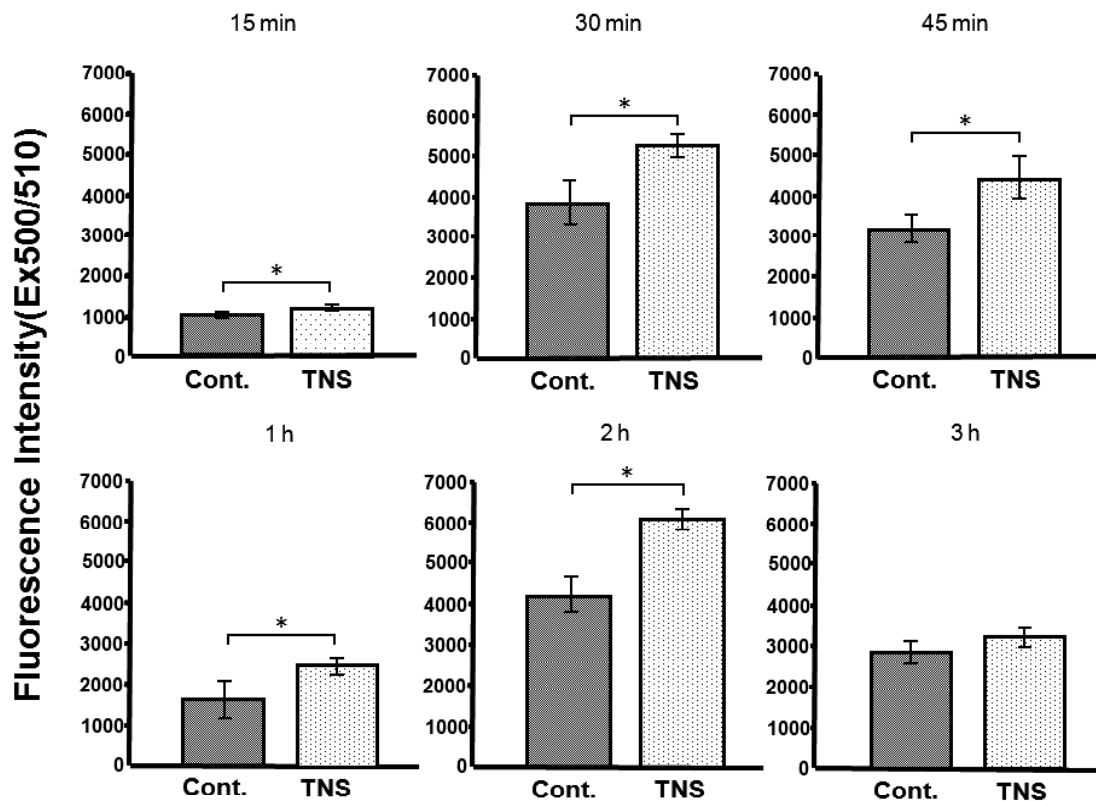


Fig. 2 The effect of TNS on the proliferation of RAECs was measured using the CellTiter-Blue™ Cell Viability assay. After incubation for 15, 30, 45, 60, or 120 min, the fluorescence intensity of the test groups was significantly higher than that of the control groups ($P < 0.05$). However, the test and control groups were no longer significantly different after 3 hours of culture.

3. Cell proliferation/viability assay

Fig. 2 shows the effects of TNS-modified on the titanium surface on the proliferation of RAECs as measured by the MTS assay. After incubation for 15, 30, 45, 60, or 120 min, the fluorescence intensity of the test groups was significantly higher than that of the control groups ($P < 0.05$). However, the test and control groups were no longer significantly different after 3 hours of culture.

4. Cell morphology

Fig. 3 shows representative fluorescence images from the test and control groups. These images revealed flat cell

adhesion in the control groups, but extended adhesion and cell outgrowth in the test group. This tendency was observed to clearly progress with time.

5. Real-time PCR analysis

Figs. 4–6 show the influence of the TNS-modified on RAEC gene expression. ICAM-1 mRNA was significantly higher in the test group than in the control group at 2 and 5 days of culture ($P < 0.05$). Von Willebrand factor and thrombomodulin mRNA expressions were also significantly higher in the test group than in the control group after 5 days of culture ($P < 0.05$).

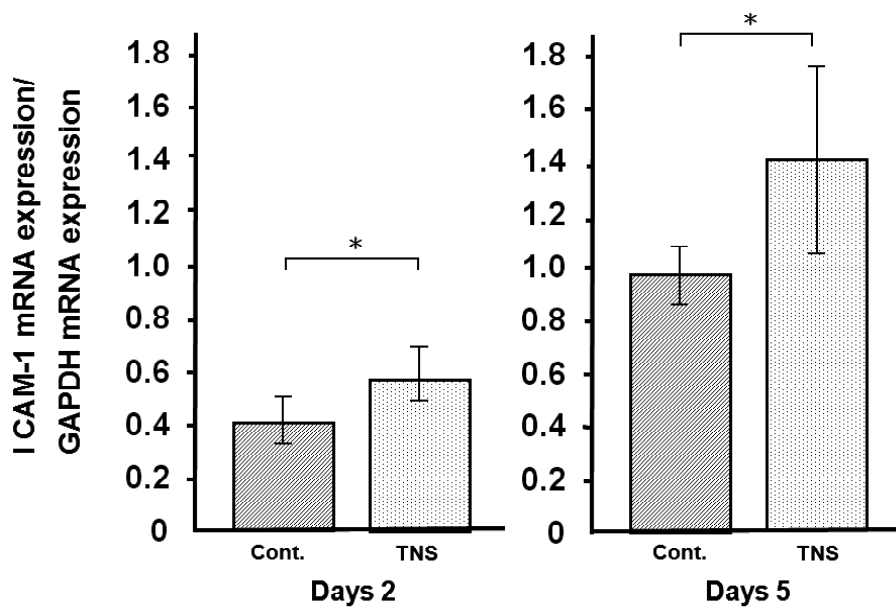


Fig. 4 ICAM-1 mRNA was quantified by real-time RT-PCR following 2 and 5 days incubation on TNS-modified titanium surfaces. ICAM-1 mRNA expression was significantly higher in the test group than in the control group after 2 and 5 days of culture ($P<0.05$).

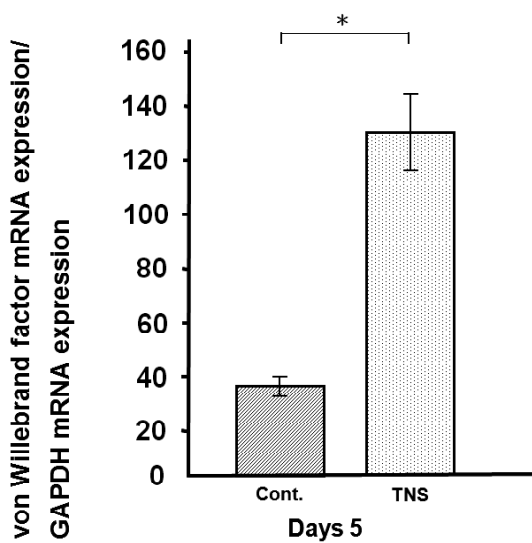


Fig. 5 Von Willebrand factor mRNA was quantified by real-time RT-PCR following 2 and 5 days incubation on TNS-modified titanium surfaces. Von Willebrand factor mRNA expression was significantly higher in the test group than in the control group after 5 days of culture ($P<0.05$).

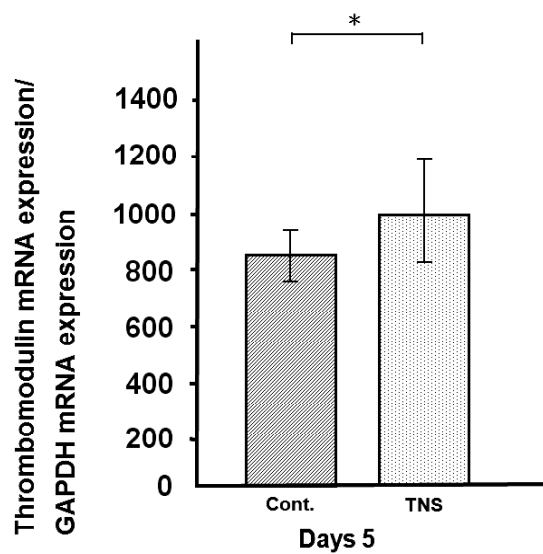


Fig. 6 Thrombomodulin mRNA was quantified by real-time RT-PCR following 2 and 5 days incubation on TNS-modified titanium surfaces. Thrombomodulin mRNA expression was significantly higher in the test group than in the control group after 5 days of culture ($P<0.05$).

DISCUSSION

Wound healing includes many processes, including inflammatory reactions, tissue remodeling, and regeneration involving angiogenesis¹⁵. The microencapsulation response to an inserted biomaterial plays an important role in the success rate of implantation¹⁶. Surface properties of dental implants were recognized early as critical factors for achieving clinical success^{17, 18}. The topographical properties of nanostructures on titanium surfaces play an important role in modulating cell response at the implant-tissue interface, which can have a large effect on tissue integration into the implant¹⁹. Recently, we showed that nanotube and TNS-modified could be fabricated on titanium metal surfaces using treatment with a 10 M NaOH aqueous solution at 30°C¹⁰, and we employed that method here to create the TNS-modified disks. Komasa *et al.* suggested that TNS on titanium surfaces leads to the regulation of osteogenic differentiation of bone marrow cells and enhances mineralization¹⁰. The angiogenic effects of a biomaterial are typically evaluated using a conventional culture of RAECs. In the present study, we report cell proliferation and gene expression of RAECs during the initial wound healing process. We found that RAEC attachment and proliferation after 15, 30, 45, 60, and 120 min of culture were increased on titanium surfaces modified with TNS. We also found that the expression of ICAM-1, von Willebrand factor, and thrombomodulin transcription factors during the early stages of the wound healing process were elevated in samples cultured on TNS-modified disks compared with unmodified titanium disks. Our results suggest the nanostructure on titanium surface can stimulate RAEC expression of angiogenic factor and adhesion molecule genes, which play an essential role in control-

ling inflammation and revascularization during wound healing after implantation.

In the present study, our results present the first report of RAEC adhesion, proliferation, and morphology on TNS-modified titanium surfaces compared with unprocessed controls. Regarding cell morphology, elongated cell shapes and cell differentiation of RAECs was observed on TNS-modified titanium surfaces compared with unprocessed titanium surfaces. Many reports state that surfaces with nanostructure features have increased surface area compared with those without such features. This increased surface area allows increased adhesion by cells such as osteoblasts and fibroblasts. Recent work has shown that treatment with an NaOH aqueous solution produces a nanoscale-rough surface¹⁰. Kasuga⁷ also reported that NaOH treatment led to the formation of a Ti-O-Na titanate layer on the Ti surface. These differences in surface nanostructure can modulate cellular osteogenic differentiation and mineralization of the titanium implant material^{2, 20}. Improvements to surface topography may be indirect, as the adsorption of proteins or ions may act as a bridge between the nanosurface structure and the cells²¹. In fact, it suggests the interdependence between mechanical surface reforming and chemical surface reforming by NaOH treatment on titanium surface leads to induce cell attachment. Thus, the altered surface energies of materials with nanostructures have been implicated as a novel feature that promotes tissue growth²² because of increased adsorption of select proteins compared with microscale-feature materials. Adsorption of select proteins can subsequently guide the adhesion, among other functions, of cells on the implant material surface^{23, 24}. A number of studies have demonstrated improved cell adhesion and proliferation on nano-modified sur-

faces that could be beneficial for various tissue applications, including bladder, bone, vasculature, and nervous system^{23,25-32}. TNS-modified on titanium surfaces in a previous study demonstrated regulation of the osteoblastic differentiation of bone marrow cells and enhanced mineralization. In the present study, TNS-modified included nanonodules of about 100 nm diameter on the titanium surfaces. Mathew *et al.* reported that mesenchymal stem cells were induced towards an osteogenic lineage on titanium surfaces with 100–120 nm features³³. In the present study, we found similar results for rat endothelial cells. Even at short times, the response of ECs to different Ti surfaces differs slightly from that of osteoblasts investigated in previous studies. To induce stable wound healing after implantation, the early response of ECs is important for treatment. Thus, our findings provide evidence that TNS-modified titanium surfaces may promote neovascularization in the wound healing phase after implantation. Also, the formation of a capillary network on the RAECs grown on TNS-modified titanium surfaces was observed after 6 days of culture using an *in vitro* tube assay. Formation of new capillary blood vessels (angiogenesis) occurs in a wide range of important biological processes, including embryonic development, osteogenesis, and wound healing. Bruni *et al.*³⁴ reported the establishment of three-dimensional networks of bovine aortic endothelial cells grown within collagen gels containing growth factors. In the present study, in spite of culturing RAEC on TNS-modified titanium surfaces without growth factors, network structure was observed after 6 days of culture. Therefore, the TNS-modified may act like a growth factor for RAECs, leading to improved tissue regeneration and wound healing.

ICAM-1 is an endothelial and leukocyte-associated transmembrane protein long known for its important role in stabilizing cell-cell interactions and facilitating leukocyte endothelial transmigration. In the present study, we evaluated the gene expression of these adhesive protein molecules following endothelial contact on TNS-modified titanium surfaces, as they can regulate RAEC adhesion to the implant material and the onset of inflammatory reactions³⁵⁻³⁷. ICAM-1 was highly expressed when RAEC were grown on TNS-modified titanium surfaces. It seems that the topography of nanostructures stimulates expression of adhesion molecules in RAEC, thereby increasing cell attachment. An *et al.*³⁸ showed surface wettability and energy promoted the expression of angiogenic factors and adhesion molecules by human ECs. The results in the present study are also supported by a recent finding that TNS-modified titanium surfaces display high wettability¹⁰. The important roles of ECs during angiogenesis include not only building up the structure of the blood vessel endothelium, but also secreting various bioactive factors³⁸. Von Willebrand factor is produced by ECs³⁹ and mediates platelet adhesion to the vascular wall⁴⁰. Thrombomodulin, a receptor of thrombin, is constitutively expressed on the EC surface, and serves to promote wound healing via a complex mechanism involving stimulation of angiogenesis and inhibition of inflammation. Our studies show that the mRNA expression levels of von Willebrand factor and thrombomodulin were elevated after culture on TNS-modified titanium surfaces compared with unprocessed titanium surfaces. An *et al.*³⁸ also reported elevated von Willebrand factor and thrombomodulin levels in human ECs on hydrophobic materials. ECs play a central role in angiogenesis during wound healing by expressing and

healing by expressing and releasing various factors. Similarly, TNS-modified may act like a growth factor to ECs, and it also may play an essential role in controlling inflammation and revascularization.

Interestingly, TNS-modified titanium surfaces induced reproducible proinflammatory effects in RAECs. Functional molecules responsible for the interactions of RAECs with TNS-modified implant surfaces were upregulated, but the impact of this on tissue regeneration and wound healing remain to be elucidated in further studies. Titanium implants have become an essential treatment modality for reconstructive surgeries in the orthopedic and dental fields. However, there is always a clinical demand for reductions in patient morbidity and treatment complications, and that outcome reliability and treatment indications are maximized. Therefore, considerable efforts have been made to develop new technologies to modify titanium implant surfaces to assist the wound healing of hard and soft tissues. The modified methods used here are useful and easily accomplished because the required incubation in NaOH is at room temperature and requires no template⁴¹.

CONCLUSION

Our investigation of different implant surface nanostructures demonstrated that modifying the implant surface at the nanometer scale can enhance RAEC adhesion and expression of angiogenic factor and adhesion molecule genes. We conclude that further development of advanced implant materials using nanotechnology will improve tissue regeneration.

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