Application of the Skin and Bone Integrated Pylon (SBIP) with titanium oxide nanotubes and seeded with dermal fibroblasts

Maxim A. Shevtsov^{1,2}, Natalia M. Yudintceva¹, Miralda I. Blinova¹, Grigoriy P. Pinaev¹, Oleg V. Galibin², Igor L. Potokin³, Ketul C. Popat⁴, and Mark R. Pitkin^{5,6,*}

¹Institute of cytology of the Russian Academy of Sciences (RAS), St. Petersburg, 194064, Russian Federation

²I.P. Pavlov State Medical University, St. Petersburg, 197022, Russian Federation

³Research institute of highly pure biopreparations, 197110, St. Petersburg, Russian Federation

⁴Colorado State University, Fort Collins, CO 80523, USA

⁵Tufts University School of Medicine, Boston, MA 02111, USA

⁶Poly-Orth International, Sharon, MA 02067, USA

Abstract

Study Design—The feasibility and safety of in bone implantation of the skin and bone integrated pylons (SBIP) with nanotubes was investigated in vitro and in vivo in the animal model.

Background—Direct Direct skeletal attachment of limb prostheses is associated with high rate of transcutaneous infection and loosening of the fixture in the medullary canal prompting for careful assessment of various means for enhancing the skin-device and bone-device interface. The SBIP system constitutes a technological platform for different modifications being evaluated previously.

Objectives—The current study assessed the combination of nano treatment SBIP with its pre seeding with dermal fibroblasts. We hypothesized that this combination will enhance cell interaction with SBIP compared to nano treatment and the fibroblast seeding when done separately.

Methods—TiO₂ nanotubes were fabricated on the SBIP, and the fibroblasts taken from rabbit's skin were cultured on the pylons before implantation.

Results—The in vitro experiments demonstrated higher cellular density in the samples with a nanotubular surface than in the non modified pylons used as control. There were no postoperative complications in any of the animals during the 6 month observation period. Subsequent SEM of the pylon extracted from the rabbit's femur showed the stable contact between the pylon and soft tissues in comparison to control samples where the patchy fibrovascular ingrowth was detected.

*Corresponding author: Mark Pitkin, PhD. Tufts University School of Medicine, Physical Medicine and Rehabilitation, 136 Harrison Ave, Boston, MA 02111; 787-297-1204. mpitkin@tuftsmedicalcenter.org.

Conclusions—The promising results prompt further investigation of the integrative properties of the nanotextured SBIP system seeded with dermal fibroblasts and its optimization for clinical application.

Keywords

nanotubes; TiO₂; skin and bone integrated pylon; fibroblast; direct skeletal attachment

Background

The necessary condition for success of direct skeletal attachment (DSA) of limb prostheses is the formation of a reliable interface between the implant and hosting tissues. Failure to create a suitable interface of the implant and skin leads to marsupialization and periprosthetic deep infections [1, 2]. It has been demonstrated that the roughness of the surface determines the degree of cell adhesion, as well as the tissue biocompatibility of the implants [3]. To date, several approaches have been introduced for modifying the implant surface with different bioactive coatings, including fibroblast growth factor 2 embedded apatite composites [4], hydroxyapatite coatings [5, 6], and with the electrochemical methods in various modifications.

Electrochemical anodization promotes the formation of a highly ordered nanostructure of nanotubes on the surface of the implant. Several studies demonstrated promising results relating to cellular interactions with the nanotubular implant's surface, as compared to the smooth surface [7–9]. Positive effect of nano treatment on the bone device interface was confirmed in [3, 10–16]. In the work of Ruckh et al. it was demonstrated that nanotube arrays on the implant surface significantly enhance osteoblast cell adhesion, proliferation and differentiation [17]. Less reports while also with positive results are known for the effect of nano treatment on the interface of a device with the skin [18, 19].

We previously developed a system for direct attachment of limb prostheses called the *Skin and Bone Integrated Pylon* (SBIP), which, in *in vitro* and *in vivo* experiments was found to support an infection free skin and bone interface [19–25]. Recent study with the SBIP having nanotubular surface [7] demonstrated more consistent, diffuse fibrovascular ingrowth into the pores of the embedded portion of the device. Angio and osteogenesis following the implantation of the SBIP seeded with autologous fibroblasts was evaluated *in vivo* [26]. Enhanced osseointegrative properties of the pylon were observed compared to the untreated porous titanium pylon. Greater cellular inhabitation within first three weeks of implantation compared to the unmodified implants was demonstrated.

As both independent studies suggested that seeding with fibroblasts and nano treatment of the implants, if taken separately, were advantageous for the skin device and skin bone interface, the aim of the current pilot study was to evaluate the combined effect from the nano treatment of the SBIP implants with their treatment with dermal fibroblasts. In this paper we present the results of *in vitro* tests and trials with rabbits.

Methods

Development of the skin and bone integrated pylon (SBIP) with nanotubular surface

There are eight known implant systems for DSA, including the SBIP system, all of whose features are summarized in [27]. The first DSA system was applied at the Rancho Los Amigos Hospital (RLAH) to three amputees in 1977 [28]. Currently, three systems: OPRA [29], EEEF [30], and ITAP [31], have been used in human patients. The POP [32] and SBIP [20, 24] systems are being used in continuing pre clinical and pilot animal studies, and the AEAHBM [33] and UA [34] systems are being used in pilot animal studies.

The principal innovation of the SBIP system and its distinction from existing systems for DSA is the total permeability of the pylon. This provides for deep ingrowth of the hosting tissues, both of the bone and of the skin. The permeability is achieved in a composite structure consisting of porous cladding and perforated inserts [21]. Specification for all modifications of the SBIP pylons, defining the four critical parameters of particle size, pore size, porosity and volume fraction, is uniquely selected and protected by US Patent # 8257435. Durability, resistance to fatigue, safety and efficiency of the SBIP specification has been confirmed in mechanical and animal studies [19, 21, 22, 27, 35 41].

Porous titanium rods (0.3 cm diameter $\times$ 2 cm length) for the current study were sintered by ADMA Products Group, Hudson, OH, with Ti6–AL–4V ELI powders, and using 0.3 cm diameter cylindrical boron nitride molds (Payne Engineering & Fab. Co., Canton, MA). The high temperature sintering was conducted in vacuum using titanium powders sieved to (80+200) mesh. Samples were sintered at 1090°C for 4 hours which was above the beta transus temperature of 996°C. Before the sintering cycle (Vacuum Industries Super VII furnace (Centorr Vacuum Industries Inc; Nashua, NH, USA) under vacuum $3.333 \cdot 10^{-3}$ Pa), three thin (0.05 cm diameter) titanium wires Ti6–AL–4V ELI (Small Parts, Seattle, WA) were inserted inside the molds. The rods had pore sizes 40 – 100 μ m, and porosity $45 \pm 5\%$ (**Figure 1, A-C**).

Titanium nanotubular surface treatment was performed at the Colorado State University, Fort Collins, CO using a simple anodization process [42] as being described elsewhere [19]. Prior to anodization, the rods were cleaned with soap, acetone and isopropanol. A titanium foil as the anode and platinum foil as the cathode formed a two electrode cell. The cell was introduced into an electrolyte solution, prepared with diethylene glycol (DEG, 99.7%), 2% hydrofluoric acid (HF, 48% solution) and 3% de ionized water. The treatments were carried out at room temperature for 24 hours at 60 V. After anodization, the samples were rinsed with isopropanol and dried with nitrogen gas. Crystallized substrates were obtained by annealing the anodized titania nanotube arrays at 530° C in an oxygen ambient environment for 3 hours. The nanotube architecture was examined field with emission scanning electron microscope (SEM, JEOL JSM 6300). Prior to imaging at 15 kV, the substrates were coated with a 10 nm layer of gold. The fabricated nanotubular surfaces had pore diameters ranging from 50 to 250 nm (**Fig. 1, D-F**).

***In vitro* assessment of fibroblasts' interaction with the SBIP system**

In a series of *in vitro* experiments, samples of porous titanium pylons with an electrochemically modified surface were put in a non polymerized collagenous gel. The gel covered the surface of the pylon (there were 9 samples in total). Pylons with a non modified surface were used as controls (there were 9 samples in total). Following incubation with the gel, the pylons were incubated in 6% CO₂ and 37 °C for full polymerization of the collagenous gel and were then transferred to Petri dishes seeded with dermal fibroblasts (Fig. 2). The fibroblasts were obtained from rabbit derma, as described elsewhere [25]. The fibroblasts were taken from cells that had migrated from fragments of the rabbit's skin that was obtained during surgery. The skin was cut into small pieces and placed in a Petri dish with a DMEM medium, supplemented with 10% fetal bovine serum (FBS), 2 mm L glutamine and antibiotics (100 U/ml penicillin G and 0.1 mg/ml streptomycin). Cells were grown at 37 °C in a CO₂ incubator with 6% CO₂ and 90% humidity. In 3–4 weeks, the plate was covered with a confluent cell monolayer. Cells were routinely tested and found to be free of mycoplasma contamination. Before the experiments, the cells were harvested in the log phase of growth, and their viability was determined by 0.4% trypan blue exclusion.

Titanium pylons were cultured with dermal fibroblasts for various time intervals (5, 10 and 15 days) and were then removed from the gel, fixed in 4% formaldehyde (Sigma, USA). To assess the fibroblasts' interaction with the pylon, three samples from each time interval were used (9 samples in total). Cell morphology on nanotube arrays was analyzed with scanning electron microscopy (SEM) (GSM 35.7, Japan).

***In vivo* animal experiments**

The protocol of the study followed the institutional guidelines of the xxxxxxxxxxxxxx for the welfare of animals. The protocol was submitted for approval to the local ethics committee and the ethical approval was obtained.

The feasibility of the in bone implantation of the electrochemically modified SBIP system was assessed in New Zealand male rabbits. The animals (3.5 – 4 kg) were purchased from the animal nursery "Rappolovo" of the Russian Academy of Medical Sciences (RAMN) (St. Petersburg, Russia). Before the insertion of the implants into the rabbit's femur they were pretreated with fibroblasts in collagenous gel for 7 days. For anesthesia we used intravenous injection of ketamine (10–50 mg/kg) and xylazine (1–3 mg/kg) mixture.

We performed unilateral above knee amputation in six rabbits that were randomly divided into two groups (three animals each): (1) in experimental group we implanted intramedullarily the modified titanium pylon with nanotubular surface; (2) in control group we inserted pylon with non modified surface.

In all animals we performed above knee amputation described elsewhere [22]. The anterior and posterior skin flaps at the level of bony resection were marked 4 cm above the knee with a marking pen. The skin and subcutaneous tissues were incised down to the fascia, provided that the anterior flap was 2.5 cm longer than the posterior flap. The greater saphenous vein was ligated and divided on the medial aspect of the thigh. Subcutaneous tissue and fascia were divided in line with the skin and reflected proximally. Femoral vessels and nerves were

identified deep to the sartorius muscle. Posteriorly, the sciatic nerve was identified deep to the hamstrings on the adductor magnus. Nerves were gently pulled down from their muscular bed approximately 2 cm, ligated with nonabsorbable monofilament sutures, transected with a surgical knife and allowed to retract back to the muscle mass. Deep femoral vessels were divided; the artery and vein were sutured and ligated individually. Then we cut the bone with a circular saw. Muscle flaps were approximated (myoplasty). The quadriceps and hamstrings muscles were myodesed to each other in covering the bony end of the femur. During the surgery we implanted modified SBIP system with nanotubular surface immediately after the above knee amputation. The position of the pylon inside the femoral canal was monitored using fluoroscopy. The pylon was press fit intramedullarily into the residual femoral bone and the soft tissues and skin were closed over the residuum.

Radiographs (46 kV, 200 mA, 32 ms, Trophy N800 HF, Fujifilm 24*30 cm² IP cassette type C, 1 m film focus distance) were taken prior to surgery, one month after the implantation of the intraosseous component, and then monthly for up to six months. For radiography, rabbits were sedated by an intramuscular injection of xylazine (1–3 mg/kg) and ketamine (10–50 mg/kg) mixture.

Results

Attachment of fibroblasts to the nanotubular surface of the pylon *in vitro*

The fibroblasts' morphology, cellular adhesion, migration and proliferation on the SBIP with electrochemically modified surface were assessed with scanning electron microscopy (SEM). After 5 days of *in vitro* cultivation, the dermal fibroblasts on the pylons could be observed on the surface of the rods; they were adjacent to the pores covered with nanotubes by cellular bundles (**Fig. 3**). Observed with scanning microscopy, the pattern of cellular growth on the surface of the pylons resembled the one when non modified control titanium pylons were used (**Fig. 4**) [25]. As in that series of *in vitro* experiments [25], cells were seen not only on the surface of the rods but also in the interior of the pylon. On the transverse sections of the titanium pylons we could observe the fibroblast bundles throughout the section, thus proving that cells can migrate through the pores of the SBIP. Cellular density increased significantly after the 15th day of incubation (**Fig. 3**). Well spread fibroblasts formed large bundles that overlapped pores in the pylon and multilayer coatings (**Fig. 3**). The cellular density on the control titanium samples with non modified surface did not demonstrate any increase on the 15th day of cultivation.

Feasibility of in-bone implantation of the SBIP system with a nanotubular surface

The intraoperative photo of the press fit pylon with nanotextured surface is shown in **Figure 5**. There were no postoperative complications in any of the animals after surgery, nor during the follow up period of six months. There were no clinical signs of infection during the observation period, and all animals behaved normally. For a deep infection control we performed antero posterior (AP) and lateral radiographs before the operation and every month after surgery for 6 months (7 radiographs in total). To evaluate the bone infection we applied parameters of the scoring system of Schmidmaier et al. [43] which includes the following parameters: (1) periosteal reaction, (2) osteolysis, (3) soft tissue swelling, (4)

deformity, (5) general impression or destruction, (6) loosening of the prosthesis, and (7) sequestrum formation.

On the postoperational radiographs we did not observe any signs of the osteomyelitis. We did not observe osseous changes on the radiographs during the entire period of observation in the rabbits. One such radiograph, presented as an example from animal #2 is shown in **Figure 6A**. At the end of the follow up period we extracted the pylon from the femur and analyzed with scanning electronic microscopy (SEM) the following surfaces of the SBIP system (**Fig. 6**): (a) contact between the pylon and soft tissues (**Fig. 6B**), (b) cross section of the pylon (**Fig. 6C**), (c) contact between the pylon and the inner surface of the femur canal (**Fig. 6D**). After 6 months we could observe reliable contact between the pylon and connective tissue (**Fig. 6B**).

On the cross section of the titanium rod we observed ingrowth of the soft tissues into the pores of the pylon throughout the cross section (**Fig. 6C**). No zones could be found where the pylon was not in contact with soft tissues. Also, when we analyzed the surface on the pylon that was in contact with the inner surface of the femur, we observed reliable contact of the nanotubular surface with tissues (**Fig. 6D**). Subsequent analysis of the radiographs demonstrated that the pylon did not migrate within the femur canal in any of the animals, thus proving the integration of the SBIP system with surrounding tissues.

We also assessed the SEM images of the pylon's non modified surface interaction with soft tissues (**Fig. 7**). After 6 months of surgery we extracted the pylon from the femur and as in experimental group of animals we analyzed the region of the pylon in contact with soft tissues (*distal end*), with bone (*proximal end*), and the cross section. There was no difference in the soft tissue ingrowth in the distal end between modified and non modified pylons. However, when we compared the surface being in contact with bone we observed patchy fibrovascular ingrowth in control titanium samples (**Fig. 7C**). On the cross sections of the control rods we also observed the regions of the pylon that were not covered by the soft tissues.

Discussions

Positive effect on osseointegration following nano treatment of the surface of the solid implants has been reported in many studies. Lesser data are available on osseointegration with the nano treated porous scaffolds. In a study [44] such scaffolds were fabricated using both nano hydroxyapatite (nano HA) powder (20 nm average particle size) and micro HA powder. The osteoblast attachment and proliferation was greater on the nano HA scaffolds compared to similarly processed micro HA scaffolds 5 days after seeding.

Current *in vivo* study demonstrated the feasibility and safety of in bone implantation of the titanium pylon with nanotubular surface pretreated with fibroblasts in collagenous gel. The rabbits did not experience any postoperative complications (i.e., implant loosening, deep infection, bone fractions, etc.) during the entire 6 months observation period. Moreover, we observed that the SBIP reliably integrated with the surrounding tissues, thereby providing stability to the pylon in the medullary canal (**Fig. 6**). The cross section of the pylon clearly

demonstrated the ingrowth of the soft tissues into the pores of the titanium, that was similar in nature to our previous experiments with non modified pylons [22, 45, 46]. With the non modified pylons (control), following 6 months after surgery we observed the patchy fibrovascular ingrowth (**Fig. 7**). These data are in line with recent results of Farrell et al., when the authors assessed the skin ingrowth in pylons with nanotubular surface in rats [7]. The authors reported that the implants with nanotubular surface had more consistent and diffuse soft tissues attachment in comparison with the pylons without nano surface, though the period of post operative observation was limited to 6 weeks [7]. In our study we extended the period of long term implantation to 6 months and confirmed the better tissue ingrowth in experimental group of the nanotextured pylons.

The *in vitro* analysis of cells' interaction with titanium rods demonstrated that electrochemical modification of the pylon surface with nanotubes significantly increased the cellular density when compared to the non modified rods. On the 15th day of co incubation of fibroblasts with titanium rods we observed multilayer coatings on the pylon (**Fig. 3**). The fibroblasts' cellular coatings on the nanotubular surface were denser in comparison to the non modified pylons from our previous observations [25].

In our study we observed a formation of a confluent fibroblast monolayer on nanotubes, 130–140 nm in diameter. The presented data are in accord with the results by Oh [47], when it was demonstrated that osteogenic differentiation of human MSCs was highest on the 100 nm nanotubes. At the same time, the rates of adhesion were maximal at 30 nm. Wang et al. investigated the effects of TiO₂ nanotubes with different diameters (30 nm, 70 nm and 100 nm) on the biological attachment mechanism of implants to bone in minipigs by studying the gene expression and bone formation around the implants [48]. The authors reported a significant increase in bone implant contact (BIC) and gene expression (i.e., alkaline phosphatase (ALP), osterix (Ox), collagen I (Col I) and tartrate resistant acid phosphatase (TRAP)) in bone attached to implants with TiO₂ nanotubes, especially with 70 nm diameter. Probably, the processes of cell adhesion, migration, proliferation and differentiation not only depend on the size of the pores of the nanotubes, but also on the particular cell biology and the chemical modifications of nanotubes. Thus, Sul [3] demonstrated the importance of the roughness of the pylon surface for the proper cell adhesion over smoother surfaces. The authors proved the superior bone response from fluorinated TiO₂ nanotube implants, despite their low roughness values. In experiments *in vivo* on rabbits, the new bone formation in case of nanotubes was increased from 57.5% to 65.5%, whereas the osseointegration strength was also elevated (from 29 to 41 Ncm) [3]. In the studies by various groups the importance was emphasized of the chemical modifications of nanotubes, especially by including fluoride (F) and strontium (Sr), and their influence on bone formation [3, 14, 49].

Conclusions

In summary, in the presented work we demonstrated the feasibility of direct skeletal attachment of the SBIP system with nanotubular surface *in vitro* and in animals. The promising results beg further investigation of the integrative properties of the nanotextured SBIP system and its optimization for clinical applications.

Limitations of the current study include a small number of animals and the fact that the implantation was performed subcutaneously. The authors will address these limitations in their further research.

Acknowledgments

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Clinical Relevance

The study is devoted to the development of more safe and efficient technology of direct skeletal attachment of limb prostheses aimed in improving quality of life of people with amputations.

Figure 1.

A, B, C – SBIP samples images with magnification x100; x500; x1,000 of SBIP pylon surface before nano treatment (anodization). **D, E, F** - images after nano treatment x1,000; x10,000; x65,000.

Figure 2.
Light microscopy photo of the SBIP in the collagen gel with autologous fibroblasts (shown with white solid arrows) on the 10th day of cultivation. Scale bar – 1 cm.

Figure 3.

Scanning electron microscopy (SEM) of the SBIP with titanium oxide (TiO_2) nanotubes on the 5th, 10th and 15th day of incubation *in vitro* with dermal fibroblasts in collagen gel. On the 5th day of cultivation fibroblasts formed bundles over the pores of the pylon (red arrows). On the magnification cells attached to nanotubes by forming filopodia (red solid arrows). On the 10th day fibroblasts formed thick cellular bundles with firm attachment to the nanotubes (red arrows). On the 15th day cells overlapped the pores in multilayer coatings (red arrows) and nanotextured surface of the pylon could barely be detected. Scale bar for the left column – 10 μm , for the right column – 1 μm .

Figure 4. Scanning electronic microscopy of the non modified SBIP with dermal fibroblasts on the 5th day of cultivation *in vitro*. Cells formed bundles over the pores of the pylon (shown by red solid arrows). Scale bar – 10 μ m.

Figure 5. Intraoperative photo of the SBIP with TiO₂ nanotubes implanted into the femur after the above knee amputation (animal #2). Prior to insertion into the femur the pylon was pretreated with autologous rabbit's fibroblasts in collagenic gel for 7 days. Scale bar – 1 cm.

Figure 6.

Animal #2 with intramedullary inserted SBIP system with TiO₂ nanotubes 6 months after surgery. **(A)** Radiograph of the in bone implanted SBIP. Scale bar – 1 cm. **(B)** Scanning electron microscopy (SEM) of the extracted pylon. Presented is the surface of the pylon in contact with soft tissues of the stump. Scale bar – 10 μm. **(C)** SEM of the pylon cross section. The dramatic ingrowth of the soft tissues inside the pores of the pylon is observed. Scale bar – 10 μm. **(D)** SEM of the surface of pylon in contact with bone tissues. All the area is covered with tissues. Scale bar – 10 μm.

Figure 7.

Animal #3 with intramedullary inserted SBIP system without nano surface 6 months after surgery. **(A)** Intraoperative photo of in bone insertion of the pylon. **(B)** Radiograph of the in bone implanted SBIP (Scale bar – 1 cm) with subsequent scanning electron microscopy (SEM) of the extracted pylon. Presented is the surface of the pylon in contact with soft tissues (*distal end*), cross section of the pylon, and surface in contact with bone (*proximal end*). Scale bar – 10 Xm. **(C)** Magnified SEM image of the pylon's cross section with patchy fibrovascular ingrowth. Scale bar – 10 μ m.