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Titanium implants alter endothelial function and vasoconstriction via a protein kinase C-regulated pathway

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Abstract

The application of titanium (Ti) alloy in joint prostheses is a good choice in orthopedic reconstruction. An elevated serum concentration of Ti has been shown in the patients with loosened knee prostheses. The precise actions of elevated Ti on the circulation remain unclear. In this study the maximal contractile responses elicited by phenylephrine in the aortas of rats 4 weeks after Ti alloy implantation and in cultured rat aortas treated with a soluble form of Ti for a period of 18 h were significantly decreased as compared with controls. Aortas isolated from rats with Ti alloy implants or aortas treated with a soluble form of Ti had enhanced protein expression of endothelial nitric oxide synthase (eNOS) and protein kinase C (PKC)-α and enhanced phosphorylation of extracellular signal-regulated kinase (ERK) 1/2. Treatment of human umbilical vein endothelial cells (HUVECs) with a soluble form of Ti for 24 h dose-dependently increased eNOS protein expression. Short-term treatment of HUVECs with Ti for 1 h effectively enhanced the phosphorylation of eNOS, PKC (pan) and ERK1/2. PKC inhibitors RO320432 and chelerythrine effectively inhibited Ti-enhanced phosphorylation of eNOS and PKC (pan). These results indicate that Ti in the circulation may alter endothelial function and reduce vasoconstriction.

Keywords: Titanium alloy implants; eNOS; Aorta; Endothelial cells

1. Introduction

Titanium (Ti) occurs widely in the natural environment and is detectable in many kinds of foods. The specific properties of resistance to corrosion and inertness allow many metallurgical applications of Ti in daily life. The introduction of Ti into orthopedic joint prostheses and the development of reconstructive techniques, as well as refinements in biomaterial science, have made total joint arthroplasty a breakthrough this century in the treatment of patients with severe arthritis. However, the wide use of joint prostheses has led to several post-operative complications, including regional osteolysis and loosening of the prostheses. These are difficult challenges to orthopedic surgeons. The dissemination of soluble metallic corrosion products and particulate metallic wear debris after long-term implantation has been shown to play an important role in prosthesis-related late complications [1,2]. It has been demonstrated that wear metal particles are disseminated in systemic organs such as the liver, spleen and para-aortic lymph nodes of patients with prostheses [3]. Metal particles generated at non-bearing surfaces in hip arthroplasty have

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been found to accumulate in the liver and spleen [4]. Some studies have suggested that metals dissolve, circulate in the body fluid and accumulate in remote organs during total hip arthroplasty [1,5]. Kasai and colleagues have indicated that approximately one-third of patients with Ti alloy spinal implants analyzed exhibited abnormal serum or hair metal concentrations at a mean time of 5.1 years after surgery, and these metals might travel to distant organs after dissolution from spinal implants [6]. The long-term pathophysiological effects of metals accumulated in organs are unknown. In our previous studies we found that elevated blood levels of Ti in patients with loosened Ti–6Al–4V alloy prostheses [7] and in rats with Ti–6Al–4V alloy implants [8]. However, the precise action and mechanism of elevated concentrations of Ti on the circulation have not been well studied up to the present.

Endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO) plays a key role in cardiovascular homeostasis [9,10]. The generation of NO by the vascular endothelium maintains a continuous vasodilator tone that is essential for the regulation of blood flow and blood pressure. The hypothesis of this study is that soluble forms of Ti may act on the circulation and further alter endothelial eNOS expression and function and blood vessel constriction. In the present study, therefore, we intend to explore the in vivo effects of Ti alloy implants on rat blood vessels and in vitro effects of soluble forms of Ti on organ cultures of isolated rat aortas and cultured human umbilical vein endothelial cells (HUVECs). We performed experiments to examine the effects of Ti on phenylephrine-induced blood vessel constriction and expression of eNOS and signaling proteins regulated by it in rat thoracic aortic rings. In addition, we investigated the cellular effects of Ti on the expression of eNOS and signaling proteins regulated by it in human endothelial cells.

2. Materials and methods

2.1. The insertion of titanium alloy implants

Male Wistar rats (200–250 g) were purchased from the Animal Center of the College of Medicine, National Taiwan University, Taipei, Taiwan. The Animal Research Committee of National Taiwan University, College of Medicine, approved the study in accordance with the guidelines for the care and use of laboratory animals. The insertion of titanium alloy implants, of a material similar to a clinical Ti alloy prosthesis, was done under pentobarbital anesthesia. The disc-shaped implant had a diameter of 5 mm and was 2 mm thick. All implants were rinsed in 70% ethanol in water and were then autoclaved. The implants were placed in the abdominal wall between the peritoneum and the rectus muscle. After 4 weeks the animals were killed under pentobarbital anesthesia to isolate the aortas.

In some experiments titanium dioxide (TiO_2, Aldrich) in sulfuric acid was prepared as a soluble form of Ti. TiO_2 was dissolved in 1.25 mM sulfuric acid as a stock solution at a concentration of 10 mM and was adjusted to pH 7.0.

2.2. Rat thoracic aortic rings and vasoconstriction study

Aortas were isolated from rats with titanium alloy implants and sham control rats under pentobarbital anesthesia. The vasoconstriction of aortic rings was measured by a method previously described [11]. Rings, 4–5 mm wide, of thoracic aortas were suspended between two hooks connected to a transducer (Grass FT.03) for the measurement of isometric force. Rings with an intact endothelium were used. The rings were suspended in 10 ml organ baths containing oxygenated (95% O_2 + 5% CO_2), warmed (37 °C) Krebs solution containing 118.3 mM NaCl, KCl 4.7, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$ and 11.1 mM glucose. Basal tension was set at 1.0 g. The rings were washed three times for 20 min each time before a concentration–contractile response curve to phenylephrine (0.01–10 μM) was obtained. The tension was recorded through an isometric transducer on a Biopac MP 100 data acquisition system with analytic software (AcqKnowledge, Biopac Systems Inc., USA), the output of which was printed on a HP deskjet 500C. In some experiments aortic rings isolated from normal rats were cultured in organ culture Petri dishes (Falcon) with sterile Dulbecco-modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic solution at 37 °C. After 18 h rings, treated or not with a soluble form of Ti were prepared for vasoconstriction experiments. For immunoblotting studies the rings were homogenized in buffer containing 20 mM HEPES, 0.25 M sucrose, 0.5 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg ml^{-1} leupeptin and 10 μg ml^{-1} aprotinin, pH 7.5, and centrifuged at 10,000 rpm for 20 min at 4 °C to remove debris.

2.3. Cell cultures

2.3.1. Human endothelial cells

HUVECs were cultured as previously described [12,13]. Cells were seeded at a density of 1×10^5 per 75 cm^2 flask in medium 199 (Gibco, Grand Island, NY), supplemented with 20 mM HEPES, 100 μg ml^{-1} endothelial cell growth substance (Collaborative Research Inc., Bedford, MA) and 20% fetal calf serum (Gibco). The cultures were maintained at 37 °C with a 5% CO_2, 95% air atmosphere. Subcultures were performed using trypsin–EDTA. All media were filtered and supplemented with 5 U ml^{-1} heparin, 100 IU ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin. The medium was changed every 2 days. The endothelial cell monolayers were identified by the presence of factor VIII-related antigen (Histoset Kit, Immunolok, Carpinteria, CA) and the typical "cobblestone" appearance. Actively growing endothelial cells from passages 3–5 were used for the experiments.

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