Colloids and Surfaces B: Biointerfaces 159 (2017) 97–107

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Full Length Article

Investigating *the race for the surface* and skin integration in clinically retrieved abutments with two-photon microscopy

D. Kapsokalyvas[a,1], M. van Hoof[b,*,1], S. Wigren[c], T. Chimhanda[a], H.J. Kuijpers[a], F.C.S. Ramaekers[d], R.J. Stokroos[b], M.A.M.J. van Zandvoort[a]

[a] *Department of Molecular Cell Biology, CARIM School for Cardiovascular diseases, Maastricht University Medical Center, UNS 50, 6229 ER Maastricht, The Netherlands*
[b] *Department of Otorhinolaryngology and Head and Neck Surgery, Maastricht University Medical Center, PO Box 5800, 6202, AZ, Maastricht, The Netherlands*
[c] *Cochlear Bone Anchored Solutions, AB, Mölnlycke, Sweden*
[d] *Department of Molecular Cell Biology, GROW-School for Oncology and Developmental Biology, Maastricht University Medical Center, PO Box 616, 6200 MD Maastricht, The Netherlands*

ARTICLE INFO

Article history:
Received 3 April 2017
Received in revised form 14 July 2017
Accepted 26 July 2017
Available online 27 July 2017

Keywords:
Hydroxyapatite
Two-photon microscopy
Skin integration
Abutment

ABSTRACT

Bone conduction hearing implants can rehabilitate some types of hearing loss. A hydroxyapatite (HA)-coated skin-penetrating abutment was developed to allow for soft tissue preservation and increased skin-abutment adherence. Inflammation is thought to relate to bacterial infection of pockets around the abutment. Upon integration, the host's ability to cover the abutment surface ("race for the surface"), and thus control and prevent competitive bacteria from colonizing it, is improved. However, the attachment mechanisms behind it are not clear. In this study, we applied two-photon microscopy to visualize tissue attachment on abutments retrieved from patients. Skin integration markers were validated and applied to four HA-coated abutments. Evidence of skin integration was found, including the presence of hemidesmosomes, a basement membrane, dermal collagen and vascularization. Cases with clinical signs of severe inflammation and evident biofilm formation showed limited skin integration based on these indicators, confirming the applicability of the "race for the surface" model.

1. Introduction

Percutaneous implants are associated with frequent inflammation throughout all branches of medicine [1,2]. Any implant that breaches the barrier of the skin, subjects its host to increased influence of the external environment [3]. A point of entry for pathogens is created, challenging the host's immune defense [4,5] and wound healing response [6]. In this respect, the semi-implantable bone conduction hearing implant (BCHI) is no exception [7]. The implant system consists of a screw-shaped intraosseous implant that integrates with the skull bone (osseointegration [8]) and an abutment that permanently penetrates the skin and attaches to an external sound processor. This system rehabilitates patients with a specific type of hearing impairment [9]. However, inflammation of the tissue surrounding the skin-penetrating abutment affects approximately 30% of patients within two years [10].

Conventionally, percutaneous BCHIs were placed using extensive soft tissue reduction around the all-titanium abutment to minimize the amount of inflammation by stabilizing the skin [7] and preventing deep peri-abutment skin pockets to form. However, the removal of subcutaneous tissues is associated with other complications, such as numbness and cosmetic issues [11]. A hydroxyapatite(HA)-coated titanium abutment was developed to mitigate the need for soft tissue reduction [12]. Previous investigations have shown that, in contrast to the traditional titanium abutments, HA allows attachment of tissue from the host [13]. A tight dermal adherence has been demonstrated in animal models [12,14]. A tight seal between the abutment surface and adjacent soft tissues [15,16] prevents bacteria from accumulating at the skin-abutment interface ('*pocket formation*'). As a result, bacteria are inhibited from colonizing the abutment surface before the host integrates with it (a model referred to as the '*race for the surface*' [17]). If the host is successful in forming a structural connection with the abutment, this mechanical barrier in association with its immune response may protect the abutment surface from continuous attempts of invasion by bacteria [13].

* Corresponding author.
E-mail address: marc.hoofvan@mumc.nl (M. van Hoof).
[1] These authors have contributed equally to this investigation.

http://dx.doi.org/10.1016/j.colsurfb.2017.07.072
0927-7765/

The competition between nutrient driven bacteria and the host's cells to populate a surface is complex and dynamic [18]. Both modify their behavior in response to the actual situation (e.g. available nutrients vs. breach of integrity of the host) and each other (e.g. infection, evasion [19,20], bacterial tolerance of the host [20,21], tolerance to antibiotic treatment [22] or eradication). Both also change their behavior over time (e.g. biofilm formation vs. different phases of the immune response, wound closure and a foreign body reaction [23]). The host response includes many different solitary and collaborating cell types which are either blood-borne (e.g. neutrophils, macrophages, fibrocytes [24]) or locally present in the skin [4] (e.g. keratinocytes, corneocytes, fibroblasts and dendritic cells). The balance is further complicated by bacterial colonies that can be composed of multiple species with different degrees of virulence and different modes of action (e.g. intracellular infection) [2].

The dynamics between bacteria and the host cells, which influences the "*race for the surface*", is often investigated in a simplified manner using a combination of cell line(s) and selected bacterial species *in vitro* [25]. As long as the complexity of the process is not sufficiently reflected in these experiments, the ability to translate in vitro research to clinical practice is restricted. To date, no artificial implant has achieved an incidence of inflammation as low as physiological, soft tissue penetrating systems. Importantly, the challenge of a successful abutment-skin interface does not only lie in creating a mechanical seal or achieving integration, but also in reestablishing such an interface over a lifetime (e.g. in response to infections, skin tearing, abutment changes etc.). Percutaneous abutments retrieved from patients, in contrast to common animal models, represent the actual, prevalent states in which they can be found in daily clinical practice. The multitude of known and unknown variables [6] that influence the skin-abutment interface converge to and determine these clinical states (e.g. skin integration, fibrotic encapsulation, bacterial colonization, etc.). Therefore, one of the objectives of this *ex vivo* clinical investigation was to assess the feasibility to study the skin-abutment interface in high detail in devices that have been in situ.

In a previous scanning electron microscopy (SEM) study [13], it was shown that HA-coating of an abutment results in almost full tissue coverage with an arrangement that suggests direct attachment. However, SEM did not allow to specifically analyze the cellular attachment components in the abutment-tissue interface, and therefore elucidating the mechanisms of attachment was not possible. Conventional histopathologic sample preparation and examination is complicated because of the hardness of titanium, which commonly complicates producing thin enough sections for proper subcellular resolution. While methods exist to produce thin sections, the required use of embedding materials restricts the choice of stains. By using such an approach in an animal study Larsson et al. [12] reported dermal adherence to the HA-coated abutments, limited pocket formation and epidermal downgrowth as compared to all-titanium abutments, showing a proof of concept of skin integration of HA-coated abutments.

The method proposed here combines two-photon laser scanning microscopy (TPM) with a specific set of markers to represent skin integration and 3D imaging methodology to analyze the full-length of the abutment-tissue interface. TPM has been applied successfully in imaging human skin ex vivo [26] and in vivo [27–30]. It features the advantage of cross-sectional deep tissue penetration without destructive sectioning. Cellular and connective tissue morphology can be locally visualized with high contrast, even without labeling [31]. Using TPM we aimed at visualizing the structures that can adhere to the HA surface. We hypothesize that hemidesmosome complexes relevant for cell-substratum adhesion [32] and the creation of a basement membrane are the mechanisms that are used by basal keratinocytes, possibly in conjunction with other cells, to attach to the surface of an HA-coated abutment. To visualize epidermal attachment, integrin-α6 was labelled, a protein complex used by basal cells to form hemidesmosomes which connect the epidermis to the basement membranes [33]. Collagen IV is expressed in the basement membranes of the skin that interconnects the epidermis to the dermis. Collagen IV is also found in basement membrane of vasculature [34] and skin appendages [35]. Visualization of dermal collagen was pursued using CNA35 (collagen adhesion protein 35), which is known to bind strongly to various collagen types, among which collagen I, III and IV [36,37]. Cell nuclei were stained using DAPI fluorescent stain, and the morphology of the nucleus was used as an indication of the cell type. Bacterial DNA can also be stained by DAPI, thus allowing the identification of biofilm on the abutment surface.

The objective of this investigation was to develop and validate the proposed methodology and investigate molecular aspects of skin integration in relation to '*the race for the surface model*' in abutments retrieved in clinical practice.

2. Materials and methods

2.1. Ethics

The procedures in this investigation were in accordance with the legislation and ethical standards on human experimentation in the Netherlands. The Dutch national competent authority, Centrale Commissie Mensgebonden Onderzoek (CCMO), was consulted about the obligation of formal competent authority and ethical approval. No formal approval was required as the study procedures were considered to be within the law (Wet op Mensgebonden Onderzoek). The anonymized materials studied were derived from patients and classified as surgical by-products. No patient identifying information was retained and samples were coded for handling. Verbal informed consent was obtained prior to removal of the sample for the specific aim. All samples were collected and analyzed at the Maastricht University Medical Center.

2.2. Sample collection

For the purpose of validating the method, reference skin biopsies using a 1 mm biopsy punch (Miltex inc., York, USA) were collected from retro-auricular skin removed during implant surgery in a patient receiving a BCHI. The surgical procedure involves punching a 5 mm hole through the full-thickness skin at the implant site, approximately 5 cm above and behind the ear, hence leaving the punch content as a waist product from which control biopsy samples were collected. The biopsy thickness (1 mm) is comparable to the thickness of the tissue that can remain on the abutment after extraction. Biopsies were immediately placed in fixation medium.

One all-titanium (Fig. 1A) Cochlear™ Baha® Abutment (Cochlear Bone Anchored Solutions AB, Mölnlycke, Sweden) and four HA-coated Cochlear Baha BA400 Abutments (top diameter 7.3 mm, bottom diameter 4.0 mm, Fig. 1B) were collected from patients scheduled for abutment change or removal. The abutments were removed by unscrewing them from the osseointegrated implant and pulling the abutment out of the skin. Demographics and clinical history were collected and all information relating to the patient was anonymized. The abutments were immediately immersed in fixation solution.

All clinical samples (abutments and biopsies) were fixed in 3.7% formaldehyde in phosphate buffered saline pH 7.4 (PBS) for 15 min, and stored in PBS with 15 mM sodium azide at 4 °C. One unused Cochlear Baha BA400 Abutment, taken directly from the sterile packaging, served as a reference sample for the HA autofluorescence signal.