Advances in the ultrastructural study of the implant–bone interface by backscattered electron imaging

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Abstract

The biocompatibility of titanium implants in bone depends on the response shown by cells in contact with the implant surface. Several developments have been targeted at achieving successful implant treatment. The aim of this study was to develop a novel preparation procedure to evaluate the bone response produced at the bone–implant interface using the technique of scanning electron microscopy with backscattered electron imaging (SEM-BSE). Dental prostheses with an SLA-modified or TOP-modified surface were implanted in a toothless part of the mandible in female pigs. The animals were sacrificed 12 weeks after surgery, at which time block specimens containing the implants were obtained. These specimens were then processed for SEM-BSE by optimizing a protocol involving chemical fixation and heavy metal staining. In addition, element distribution maps for the implant–bone tissue interface were obtained using a microanalytical system based on energy-dispersive X-ray spectrometry (EDS). This novel visualisation approach enabled a comprehensive study of the extracellular matrix and cell components of the host tissues reformed around the implant. SEM-BSE images also provided ultrastructural details of the bone cells. This technique appears to be an effective and very promising tool for detailed studies on the implant–bone tissue interface and the host response to the bone incorporation process.

Keywords: Animal study; Bone cells; Bone formation; Implants; Interface; Osseointegration; SEM-BSE

1. Introduction

The biocompatibility of titanium implants in bone depends on the interfacial response of cells in contact with the implant surface (Huang et al., 2004). The final biological response of bone to an implanted biomaterial depends on the implant’s bulk properties, its design and specific surface characteristics, which will affect the tissue repair process and the long-term behaviour of the biomaterial–tissue interface in the recipient patient (Savinaro et al., 2003). The surface properties of prostheses play an important role in how they interact with adjacent living tissue (Li et al., 2006). Several modifications have been made to implant surfaces to improve their incorporation in recipient bone tissue. In vitro and in vivo studies have revealed that the cell biology and shear strength of the bone–implant interface are influenced by the microtopography of the implant surface (Lincks et al., 1998; Deligianni et al., 2001). It is also widely known that bone calcification is preceded by the formation of an extracellular organic matrix, primarily secreted by bone cells. Bone-forming cells, active osteoblasts and osteocytes, control the synthesis of this extracellular matrix, and regulate the exchange between ions present in the bulk extracellular fluids and those present in calcified bone. Thus, a detailed in situ study and the precise visualisation of all the components of the interface between the implant surface and newly formed bone are needed to assess the bone integration process.
Several techniques have been used to assess the implant-bone tissue interface. The traditional method of histological staining followed by examination under the light microscope (LM) has enabled observation of the cell response produced during bone regeneration. Although LM has contributed substantial information, its low spatial resolution determines that no information is provided at the ultrastructural level. Transmission electron microscopy (TEM), on the other hand, has been successfully used to describe the cellular components of newly formed bone (e.g., Meyer et al., 2004). However, the technique has an inherent limitation: only soft tissue mechanically separated from the implant-bone interface that has been processed to prepare ultra-thin sections can be examined. This along with the small size of the observed area (1–2 mm²) in most cases adds to its limitations. Among the most promising tools as a complementary technique to both LM and TEM, is scanning electron microscopy (SEM) operating in backscattered electron (BSE) emission mode. The use of the SEM-BSE technique to examine the implant-bone interface was first reported by Jasty et al. (1989), Bloebaum et al. (1990) and Skedros et al. (1993a). The method was mostly used for descriptive studies performed on calcified tissue (Skedros et al., 1993a, b; Boyle and Jones, 1996; Roschger et al., 1997). Other authors have reported the use of SEM-BSE to examine fracture healing (Egger et al., 1993; Kim et al., 1992) and the bone response to the implant surface (Sul et al., 2005; Ottani et al., 2002). In addition, SEM-BSE imaging has unveiled some of the ultrastructural features of the different calcified tissues involved in the process of bone healing in studies conducted by Franch et al. (1998) and Manzanares Céspedes et al. (2004). These authors argue this technique to be one of the best methods for the morphological characterization of calcified tissues in studies designed to evaluate the fracture repair process. Despite obvious advantages of the SEM-BSE technique, so far it has only been used to describe and characterize calcified bone patterns and not for histological purposes.

The principal objective of the present study was to develop a novel protocol for preparing specimens of dental implants embedded in newly formed bone for SEM-BSE observation, for use in studies designed to assess the implant-bone interface. The idea was to achieve sufficient resolution to explore the different inorganic and biological processes of tissue healing that occur at this interface. The approach developed is also useful for the ultrastructural characterization of bone cells and their components involved in the bone integration process. We also used the energy-dispersive X-ray spectrometry (EDS) microanalytical system to prepare element distribution maps of the implant-bone tissue interface.

2. Materials and methods

2.1. Oral implants

Two types of dental implants available in the market were used: the “Phoenix” implant (Ilerimplant® S.L.-GMI S.L., Lleida, Spain), a solid screw (outer diameter 4 mm, implanted segment 10 mm) made of c.p. titanium (grade IV) with a sandblasted, acid-etched (SLA) surface (www.ilerimplant.com); and the Nobel TiUnite™ (Nobel Biocare AB, Göteborg, Sweden), also composed of titanium with a novel titanium porous oxide (TPO) coating (Hall and Lausmaa, 2000).

2.2. Animals and surgical technique

Eight female Polish Landrace pigs with an initial body weight (b.w.) ca. 250 kg were obtained from a commercial piggery and fed twice a day with a feed concentrate as rations (2% b.w.) and water ad libitum. The animal were individually caged and kept under constant conditions of light (12/12 h light/dark cycle) at room temperature, Azaperone (4 mg/kg b.w., Stressnil, Janssen Belgium) was injected intramuscularly as premedication and anaesthesia achieved with pentobarbital at the routinely used dose for second stage surgical anaesthesia. Antibiotic prophylaxis consisted of two injections (15 mg/kg b.w., amoxicillin, Clamoxyl L.A., Pfizer, UK) given 1 h before implant and continued for a further 48 h. Four animals were implanted with an SLA-modified surface implant and another four animals received a TOP-modified surface implant. Following a gingival incision, the bone was gradually penetrated using the lancet and round pilot drills to the required depth, as measured using a depth gauge. Next, a series of consecutive holes were drilled to cover a diameter appropriate for each implant and up to the required depth mark. The implants were then inserted in the prepared site until the end of the most coronal aspect of the fixture’s microthread. A contra-angle hand-piece was introduced for motorized installation with a torque strength not exceeding 40 N cm and maximum speed 15–20 rpm. If the implant was not completely inserted using the contra-angle protocol, insertion was completed using a torque wrench (maximum torque strength 40 N cm) and its screwtool. There was no need to use the screw tap in any case. The implant abutments were then sealed using the cover screws at the top of the implant holder cap. Finally, the gingiva was sutured using Maxon 3–0 absorbable suture thread (Syneture, Tycos Healthcare Group LP, USA). Surgery was performed by experienced implantologists using the techniques commonly used in humans. Treatments and experiments were conducted according to EU guidelines on the welfare of experimental animals. The study protocol was approved by the local ethics committee (application no. 40/2005, decision no. 41/2005). At week 12 post-implantation, following sedation with ketamine (10 mg/kg, i.m.) the animals were sacrificed by pentobarbiturate (Thiopental, Biochemie GMBH, Austria) overdose.

2.3. Preparing samples for SEM-BSE

Following animal sacrifice, specimens comprising the dental implants and adjacent host tissue were excised from the mandibular bone as cubes (15 mm × 15 mm × 15 mm) and immediately transferred to 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB). The samples were chemically fixed in this solution for 24 h at 4 °C. Once fixed, the samples were washed in 0.1 M PB 3 × 30 min and post-fixed in 1% osmium
tetroxide in H₂O for 24 h at 4 °C. The specimens were again washed in 0.1 M PB (3 × 30 min). Next, the samples were dehydrated in a graded ethanol series of 30% for 3 h followed by 50% for 3 h. During dehydration with the next ethanol dilution, the samples were contrasted with saturated 70% ethanol uranyl acetate overnight at 4 °C. This was followed by immersion in 96% (2 × 3 h) and finally 100% (3 × 3 h) ethanol. Next, the samples were gradually infiltrated with LR-White (The London Resin Co. Ltd., Hampshire, UK) embedding medium, first with a 1:1 mixture of LRW in 100% ethanol (3 days at 4 °C) and finally pure LRW resin (3 × 3 days at 4 °C). This step was followed by polymerisation in an oxygen-free atmosphere (24 h, 60 °C). After this, the polymerised specimen blocks were visualized by X-ray micro-radiography to determine the orientation of the implants within the tissue. This helps achieve perfect cross-sectioning through the longitudinal axis of the implant. Next, the blocks were cut using a low speed diamond saw and fine polished using grinding papers (from nr. 300 to 1200) and silicone carbide abrasive sheets with grain diameters of 15, 9 and 3 μm. Final polishing was performed using liquid diamond polishing compound on napped cloth with diamond particle sizes of 1 and 0.25 μm in an oil-based lubricant fluid. The surfaces of the polished cross-sections were post-stained with saturated uranyl acetate (in H₂O) for 30 min and with lead citrate (Reynolds, 1963) for 12 min. After washing in distilled water and air-drying, the polished block surfaces were coated with evaporated carbon.

2.4. SEM-BSE microscopy and EDS microanalysis

Prepared samples were examined in a scanning electron microscope (DSM940 Zeiss) equipped with a solid-state, four diode BSE detector plus an auxiliary EDS microanalytical system (Link ISIS Oxford). The EDS method involved qualitative and quantitative microanalysis, including element distribution mapping. In the distribution maps provided, relative concentrations are indicated by a colour scale: dark-blue represents a concentration of absolute zero and white denotes 100% absolute concentration of the respective pure-component spectrum (Kotula et al., 2003). The colour concentration scales (0–100%) for each element are provided in the corresponding images. The microscopy and/or microanalytical operating conditions were as follows: 0° tilt angle, 35° take-off angle, 15 kV acceleration potential, 6 or 25 mm working distance and 1–5 nA specimen current.

3. Results

The results presented indicate the possible application of the SEM-BSE technique to examine the implant–new bone tissue interface of the specimens prepared using the new procedure. Fig. 1 shows different aspects of the SLA-modified titanium implant–bone interface observed at 12 weeks post-implant. The low magnification view of this interface (Fig. 1a) shows the titanium implant surrounded by newly formed lamellar bone revealing a Haversian, or osteonal, bone, pattern. Details of the Haversian canal, appearing close to the implant surface, are shown in transverse view in Fig. 1b.

Note that the wall of this osteon is overlain by a layer of deposited bone tissue and osteoblast cells. According to Skedros et al. (2007), the irregular shape and relatively large diameter of this vascular channel suggests that this is the secondary osteon. The darker contrast of the lamella layer in the SEM-BSE image indicates the lower mean atomic number of the target, probably attributable to the lower content of apatite-like crystals within this structure. In the centre of this canal, a blood vessel containing erythrocytes could be distinguished. Details of the proper integration of the SLA-modified surface implant with the bone are shown in Fig. 1c. A relative abundance of osteocytes was observed in this mineralized mature bone. In some zones of the implant–bone interface, the implant surface was overlain by an extracellular matrix containing bone (osteogenic) cells (Fig. 1d and e). The calcium EDS distribution map (Fig. 1f) clearly demonstrates differences in mineralisation rates (reflected by Ca concentrations) between mineralized bone and immature bone (osteoid), as well as small amounts of calcium in the extracellular matrix.

Fig. 2 provides information on the osseointegration process produced in the implants coated with highly porous titanium oxide (TPO). The external micromorphology of this layer (before implant) can be seen in Fig. 2a. Quantitative EDS analysis of this layer revealed a titanium oxide (TiO₂) concentration (wt%) of 83.78% (n = 15, S.D. = 1.23). However, this layer was also found to contain a small quantity of phosphorus (the P concentration represented as P₂O₅ was 16.03%; n = 15, S.D. = 0.37). Our SEM-BSE images (Fig. 2b, c and e) revealed the mineralized bone to be tightly adhered to the TPO layer after 12 weeks of implant. Many of this layer’s conical- and bottle-shaped pores were filled with a matrix composed of calcium, phosphorus and oxygen (EDS map in Fig. 2d). Moreover, the EDS elemental distribution maps (Fig. 2d) distinctly show how the osseous tissue composed of calcium hydroxyapatite adheres to the TPO-modified surface, as well as revealing the appearance of the phosphorous and oxygen elements in the TPO layer (dotted line in Fig. 2d). Fig. 2f shows the occurrence of osteogenic cells close to the implant surface.

The SEM-BSE images in Fig. 3 indicate the potential of the proposed strategy to detect and characterize the bone cells present at the implant–neofomed bone interface. Even a low magnification image can provide interesting information on the contents of Haversian canal (Fig. 3a). The detailed view of the centre of the Haversian canal (Fig. 3b) shows osteogenic cells embedded in collagen fibrils. Additionally, the resolution power of this technique can render ultrastructural details of osteocyte-containing lacunae in lamellar bone. Osteocytes are star-shaped cells, and are the most abundant cell type found in bone. The most conspicuous feature of the osteocyte that appears in Fig. 3c is the round nucleus with nucleolus and endoplasmic reticulum. The next image (Fig. 3d) shows mature bone covered with an extracellular matrix of noncalcified bone. Osteoblasts were also frequently observed overlying mature bone (Fig. 3e). In close proximity to these osteoblasts, osteogenic (bone progenitor) cells could also be seen in the
extracellular matrix. Odontoblasts (Fig. 3f) were also observed in zones of osseous tissue further from the implant.

4. Discussion

The placement of an implant in the alveolar process elicits a sequence of healing events including necrosis and subsequent resorption of traumatized bone around the titanium fixture concomitant with new bone formation. While the implant initially displays mechanical stability due to contact and friction between the implant surface and the severed bone, the long-term maintenance of implant stability calls for biological attachment between the foreign body and the surrounding tissue. The precision and reliability of a histomorphometric
study of the newly formed bone depends upon the correct identification and ultrastructural characterization of all possible cellular components that play a role in the osseointegration processes. The LM lacks the resolution power required for a detailed structural analysis. The use of electron microscopy techniques could help characterize the morphological changes that occur during osseointegration.

The main electron beam in SEM causes the emission of BSE from the specimen. BSE may be used to detect contrast between areas of different chemical composition. These can be observed especially when the average atomic numbers of the components of the regions vary. Since the intensity of the BSE signal depends on the mean atomic number of the sample (Joy, 1991), the SEM-BSE technique not only serves to distinguish inorganic features,
but also offers the interesting possibility of identifying heavy metal-stained ultrastructural cell components and their micro-morphological details. The new approach presented in this study was inspired by the successful application of the SEM-BSE technique and heavy metal staining preparation procedure to the study of the microorganism–rock interface. This new research tool was reported first time by Wierzchos and Ascaso (1994). The present report describes a first attempt at applying this method to characterize the implant–bone tissue interface.

SEM-BSE imaging has the advantage of a wide magnification range determining that pieces of bone harbouring implants as small as several centimetres can be scanned. When the site of interest has been identified, it can be magnified and imaged at high-resolution close to that of TEM. Moreover, microanaly-
tical determinations in the form of both qualitative and quantitative measurements and element distribution maps can be simultaneously generated for the area of interest.

For the stability of a load-bearing implant, the formation of new bone and a mineralized matrix near the implant are key processes. The present study revealed that highly mineralized bone became closely attached to both the SLA- and TPO-modified implant surfaces. Moreover, it was observed (Fig. 2b–e) that the small pores in the TPO-modified implant surface were filled with a Ca–P–O-rich matrix, suggesting the penetration of newly formed bone tissue. This could confer these implants the desired mechanical stability. The presence of small amounts of calcium was also observed in the bone-specific extracellular matrix produced by bone cells concomitant with apatite crystal formation. These findings demonstrating calcification of the bone tissue at the implant–bone interface are in agreement with previous reports, e.g. Simmons et al. (1999). Our ultrastructural data indicate that cells with the typical appearance of well-differentiated bone cells occur in very close proximity (micrometers) to the implant surface. This observation is consistent with those described by Lavos-Valereto et al. (2001), Simmons et al. (1999) and Meyer et al. (2004) who also noted intimate contact between bone cells and titanium implants on SEM examination in both early and late post-implant stages.

5. Conclusions

SEM-BSE visualization of heavy metal-stained bone tissue is very useful for assessing the behaviour of an implant at the bone interface. SEM-BSE images of finely polished cross sections of specimens taken from the implant zone between a titanium implant and surrounding neoformed bone can provide relevant information on the presence and ultrastructural details of bone cells occurring at the implant–bone interface. Moreover, by visualizing the interactions that occur at this interface between cells and their components we will increase our understanding of the osseointegration process.

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