Microscopic view of osseointegration and functional mechanisms of implant surfaces

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ABSTRACT

Argon ion beam polishing technique was applied to prepare the cross sections of implants feasible for high resolution scanning electron microscope investigation. The interfacial microstructure between newly formed bone and implants with three modified surfaces retrieved after *in vivo* test using three different animal models was characterized. By this approach it has become possible to directly observe early bone formation, the increase of bone density, and the evolution of bone structure. The two bone growth mechanisms, distant osteogenesis and contact osteogenesis, can also be distinguished. These direct observations give, at microscopic level, a better view of osseointegration and expound the functional mechanisms of various implant surfaces for osseointegration.

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1. Introduction

The demanding orthopedic and dental implants allow the growth of osseous tissue into their surface close proximity and support connective tissue with a stable anchorage depending on the surface properties [1]. Titanium with desirable combination of biocompatibility and mechanical properties is an established biomaterial for implant application [2]. It has been shown that the healing procedure can be further improved by additional surface modifications of the implant. For example, bone apposition and soft tissue integration were found to be effectively enhanced when a sand-blasted and acid-etched surface was generated on dental implants [3,4].

Bone may form by two different processes around implants, being distant osteogenesis and contact osteogenesis, respectively. In the former case, bone formation proceeds toward the implant from existing bone (old bone), whereas, in the latter case, bone growth starts on the implant surface [5–7]. So far, bone–implant contact (BIC), defined as the percentage of dental implant surface covered by newly formed bone, is one of the critical measure used to quantify the degree of osseointegration. In practice, it appears difficult to accurately determine BIC due to the technical limitation in specimen preparation to expose the interface between bone and implant. Different BIC numbers may be generated by taking microscopic images under different magnifications. Furthermore, BIC gives no information about the type of bone formed around the implants. A careful evaluation and understanding

of the nature of the interface between the bone tissue and the implants by other means appears necessary for guiding implant design, particularly for improving initial stability and speeding up the healing procedure. Recently, focused ion beam (FIB) milling was used to prepare cross sections of dental implants and the tissue/implant interfaces. It has been proven a promising method for preparing cross sections without mechanical impact on the sample for electron microscope characterization, particularly for transmission electron microscopy (TEM) observation [8–11]. The limitation of FIB is the fact that the polished area is rather small, typical about 20 μm in edge lengths, which only cover one or two pores in case of the oxidized titanium implants. In our previous work we have developed an approach to use argon ion beam cross section polishing for preparing well-polished interfaces suitable for evaluating the interfacial microstructure between newly formed bone and implant by high resolution scanning electron microscopy (SEM) imaging [12,13]. Compared with FIB, argon ion beam cross section polishing is more convenient for preparing large polished area up to 0.1 mm in edge lengths by using one single beam. Although the sample prepared by argon ion beam cross section polishing is not suitable for TEM observation, it fits well for SEM observation to reveal the bone formation mechanisms. Furthermore, the functional mechanism of the surfaces of TiUnite®, TiXos® and sand-blasted implants during osseointegration can be elucidated, which is different from the other clinical evaluation or comparative trial of the implants with turn machined surface [14–16].

In the present study, we applied this approach to characterize the interface between newly formed bone and dental implants with three different types of surfaces. The specimens were retrieved from *in vivo*

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tests carried out by placing the implants with macroporous surfaces in mini pigs, the implants with selective laser melting (SLM) surface in beagle dogs, and the implants with sand-blasted surface in sheep. Attention was paid to reveal, on microscopic level, the two bone formation mechanisms, distant and contact osteogenesis, and their kinetics. In this way, the time dependent coherence and quality of the newly formed bone can also be characterized with sufficiently improved resolution.

2. Materials and methods

2.1. *In vivo* tests

Housing and handling of the animals used in the *in vivo* study was done in accordance with the approved ethical permission.

2.1.1. TiUnite® implants in mini pigs

TiUnite® implants with an oxidized macroporous surface (Nobel Replace Tapered Groovy RP 4.3 × 13 mm, produced by Nobel Biocare AB, Göteborg, Sweden) were used in the *in vivo* study using mini pig model. All mini pigs were at least 2 years old, thus having permanent teeth. After 4 weeks of healing the animals were sacrificed, the part of the jaw containing the implants and adjacent teeth were cut out and placed in 4% paraformaldehyde for a minimum of 1 week before further evaluation.

2.1.2. TiXos® implants in beagle dogs

TiXos® implants (TiXos® Cylindrical, Leader-Novaxa, 3.3 × 10 mm, internal hex, Milan, Italy) produced by selective laser melting technique were placed in two beagle dogs of approximately 1 year of age, weighing 10 and 13 kg, respectively. After 2 and 4 weeks of healing, the animals were euthanized with an overdose of sodium pentobarbital. The jaws were dissected and blocks containing the experimental specimens were obtained. Subsequently, the harvested tissue blocks were fixed in a 10% formol for 7 days [17].

2.1.3. Implants with sand-blasted surface in sheep

Implants with sand-blasted surface (Brånemark system Mk III RP 4.0 × 8.5 mm, REF 28891, produced by Nobel Biocare AB, Göteborg, Sweden) were used in the *in vivo* study using a sheep model. The implants were placed in the pelvis. After 2, 4 and 8 weeks of healing, the animals were sacrificed, the part of the pelvis containing the implants and bone were cut out and place in 4% paraformaldehyde for a minimum of one week before further evaluation.

2.2. Exposing the bone–implant interfaces by argon ion beam polishing

Each specimen containing one implant was infiltrated with methacrylate, polymerized (resin) and cut longitudinally in the mesio-distal direction through the middle of the implants using a diamond saw.

For each half of the specimen, prismatic blocks with approximate dimensions 1 × 5 × 5 mm³ containing a region near the middle of the implant was cut using a low-speed saw and diamond wafering blades. These blocks were mechanically polished with 400 grit silicon carbide paper and then fixed on the sample holder of the cross-section polishing apparatus using carbon paint (Conductive Carbon Cement, Plano, Wetzlar, Germany). Argon ion beam cross section polishing was performed using SM-09010 Cross-section Polisher (JEOL, Tokyo, Japan) at accelerating voltages in the range of 4–4.5 kV with beam currents between 70 and 90 μA for 20–24 h. A precut sample block with mechanically polished surfaces was covered with a shield plate, which stopped half of the argon ion beam. Only an approximately 75 μm wide part of the sample protruded from the cover. This part was slowly milled by the argon ion beam, leaving behind a well-polished surface at the position of the edge of the shielding plate.

2.3. Microstructure characterization by scanning electron microscopes

Argon ion beam polished surfaces were studied using a JSM-7000F (JEOL, Tokyo, Japan) field emission scanning electron microscope equipped with an energy dispersive X-ray (EDX) spectrometer (Oxford Instruments, Abingdon, UK). Accelerating voltages in the range of 5–10 kV were used for secondary electron (SE) and backscattered electron (BSE) imaging. EDX spectra were recorded at 10 kV.

3. Results

3.1. Microstructure of the implant surfaces

The TiUnite® implant with macroporous surface has many open pores with diameter in the range of 1–7 μm. No obvious cracks or other irregularities were observed under SEM, see Fig. 1a. The TiXos® implant surface is shown to be rich in micron-sized concavities that extend beneath the surface and interconnect through packing voids, Fig. 1b. The sand-blasted implant surface is rough with 1–20 μm ditches, as the SEM image shown in Fig. 1c reveals.

3.2. Interfacial microstructure after argon ion beam polishing

The sample preparation sequence by argon ion beam polishing is shown in Fig. 2. Fig. 2a shows the half of the specimen before cut. After cutting prismatic blocks containing a region near the middle of the implant was selected for argon ion beam polishing, see Fig. 2b. Fig. 2c shows the area after argon ion beam polishing. Usually the argon ion beam polished section includes one or two flanks of the thread and the region in between the flanks. Before ion beam polishing on mechanically polished surface it was difficult to distinguish the different microstructure features across bone–implant interface by SEM. After argon ion beam polishing, the microstructure of the implant,

Fig. 1. SEM images of different implant surfaces. (a) TiUnite® implant surface. (b) TiXos® implant surface. (c) Sand-blasted implant surface.

Fig. 2. Sample preparation sequence: (a) half of the specimen fixed in resin; (b) prismatic blocks containing implant and bone; (c) polishing area and (d) SEM image of polishing area (marked by white frame).

bone and the interface between them can be clearly revealed by SEM in back scattered electron mode, see Fig. 2d.

3.3. Implants in mini pigs

Under high magnification the oxide layer on the TiUnite® implant surface, resin and bone can be clearly distinguished in SEM images shown in Fig. 3. The thickness of the oxide layer is in the range of 4–8 μm. Pores with different sizes in the range of 1–7 μm are distributed unevenly in this oxide layer. There is a gap between the implant and the bone, which an artifact formed during the sample preparation. Its formation may be influenced by fixation, dehydration, and resin embedding [18]. The inset images in Fig. 3a show the elemental maps revealing the distribution of titanium (blue), oxygen (white), calcium (green) and phosphorus (red) obtained by the EDX analysis. A dense layer of bone is formed on the implant surface and even following the contours of the open pores. The density of bone was found to be different in different regions near the implants, see Fig. 3b. The bone around the immediate vicinity of the implant thread and old bone appears to be denser than that of the newly formed one. Between the old bone and the new bone a cement line matrix can be distinguished, and the osteocyte lacunae (filled with resin during sample preparation) can also be found in the new bone area, see Fig. 3c. Fig. 3d shows a gap between the implant and the bone, where the fibrous bone has been pulled out from the surface pores of the implant because of the shrinkage during sample preparation.

3.4. Implants in beagle dogs

Fig. 4 shows the SEM images taken on implant–bone interface after implementation of the implants with selective laser melted surface in

Fig. 3. SEM images taken on ion beam polished cross section revealing the bone–implant interface with TiUnite® implant surface in mini pigs: (a) bone deposited on implant surface and elemental maps showing the distribution of titanium (blue), oxygen (white), calcium (green) and phosphorus (red); (b) new bone with apparent lower density growing toward the implant; (c) the cement line (indicated by white arrows) separates old and new bone; (d) the pull-out of fibrous bone tissue from the surface pores inside the gap between the implant and the bone.

Fig. 4. SEM images take on ion beam polished cross section revealing the bone–implant interface with selective laser melted surface in beagle dog: (a) the gap between implant and bone (2 weeks); (b) the fibrous tissues attached on the implant surface (2 weeks). (c) The cement line between new bone and old bone (indicated by white arrows, 2 weeks). (d) Close contact established between bone and implant (4 weeks).

beagle dog. After two weeks the osseointegration is not established, but the new bone formed by two kinds of osteogenesis can be distinguished. A layer of deposited bone with fibrous structure can be observed on implant surface, see Fig. 4b, and bone also grows from the old bone toward the implant. The cement line matrix between the old bone and the new bone can be observed in Fig. 4c. After four weeks of healing the osseointegration is established, see Fig. 4d. The improved mineralization has turned the new bone to have increased density and to reveal a granular structure, see the inserted image in Fig. 4d.

3.5. Implants in sheep

Fig. 5 shows the bone–implant interfaces after implantation of the implants with sand-blasted surface in sheep for two, four and eight weeks. The osseointegration is not established after two weeks, but a layer of deposited bone can be observed on implant surface, see Fig. 5a. After four weeks the osseointegration is established, and the

newly formed bone is in coherence with implant, see Fig. 5b. The bone became denser after healing for eight weeks, see Fig. 5c.

4. Discussion

A microscopically rough surface on implant is more favored than a smooth surface as it increases bone anchorage and reinforces the biomechanical interlocking of bone with implant [19,20]. Numerous reports have demonstrated that implants with micron-scale topography enable rapid and increased bone accrual or bone implant contact [21–27]. There are several processes that can be used to achieve implant surfaces with micro-scale topography. Surfaces on TiUnite®, TiXos® and sand-blasted implants are three typical types of implant surfaces that were investigated in this work. The fact that these implants can promote osseointegration at different levels has already been an indisputable fact [14–16,26,27].

Fig. 5. SEM images take on ion beam polished cross section revealing the bone–implant interface with implant with sand-blasted surface in sheep: (a) deposited bone on sand-blasted implant surface, after 2 weeks. (b) Bone is in close contact with the implant surface, after 4 weeks. (c) Denser bone formed after 8 weeks.

By using conventional evaluation methods, e.g., histomorphometrical evaluations, removal torque tests, and success and failure criteria, a series of parameters such as BIC, removal torque and implant survival rate can be revealed. These parameters are proofs of the establishment of osseointegration; they, however, barely give an insight to the progress and mechanism of bone formation around implants. A direct electron micrographic observation can provide more information about bone–implant interface. For example, using fluorochrome labeling D.A. Puleo indicated that bone grew in two directions, and bone extending away from the implant formed at a rate about 30% faster than that of the bone moving toward the implant [24]. P. Thomsen et al. observed two types of bone, calcified bone tissue and dense amorphous bone, near implant surface [28]. Shah et al. established a multi-scale characterization approach including light optical microscopy, micro-CT, SEM and transmission electron microscopy to characterize a functionally graded bone–implant interface at the ultrastructural level [29]. Because of the complexities of the *in vivo* environment and technical limitations in specimen preparation, the bone–implant interface including bone formation progress, bone types, and bone structure changing have not yet been fully and clearly characterized.

By using argon ion beam polishing technique for sample preparation for SEM investigation it appears feasible to reveal in much more details the formation of two types of bones, i.e., the one that grows toward the implant and the one deposited on the implant surface. The former approves the distant osteogenesis mechanism, by which the newly formed bone grows toward the implant from the old bone. In this case, it appears that the old bone provides the osteogenic cells that lay down the matrix to encroach the implant leaving many osteocyte lacunas in newly formed bone. The latter approves the existence of the contact osteogenesis mechanism, by which a dense layer of bone is formed immediately on the implant surface, possibly via two peri-implant healing processes, namely osteoconduction and *de novo* bone formation, suggested early by J.E. Davies [5,6]. Osteoconduction can generate the knock-on effects on implant surface by recruitment and migration of osteogenic cells by the initiation of platelet activation to implant surface, while *de novo* bone formation results in a mineralized interfacial matrix on implant surface. Besides, many reports have stated that contact osteogenesis process seemed to be dependent on the surface roughness [5,6,30], because roughness can increase possibilities for cell fibrins to attach to the implant surface and assist bone cell migration. In the later stage of osseointegration, new bone formed by distance osteogenesis and contact osteogenesis merged together to establish tightly fixed interface between implant and bone. The direct observation of bone formation indicates these two osteogeneses start early and quickly, even in the first two weeks the different bone structures can be identified and the osseointegration can be well established within four weeks.

The bone–implant interface can be characterized in microscopic level by careful SEM investigation on well-polished surface by argon ion beam polishing. The macroporous surface of TiUnite® implant provided many anchorage points for osteogenic cells at the early time of bone formation, and the new fibrous bone also grew into the pores on the TiUnite® implant surface. These fibrous bones root deeply into pores and tightly fixed the implant and the bone. The TiXos® implant surface rich in micron-sized concavities that extend beneath the surface and interconnect through packing voids provided anchorage points for osteogenic cells in similar way as TiUnite® implant. The rough sand-blasted surface enabled the bone deposition that became already visible after two weeks. In four weeks the deposited bone and in-grown bone have merged together and became coherent along the sand-blasted surface. After eight weeks healing the bone density has increased sufficiently so it appears difficult to distinguish the newly formed bone and old bone. The macroporous surface, SLM surface and sand-blasted surface are three kinds of microscopically rough surface on implant, but these implant surfaces express their superiority multiply; it is difficult to perorate which surface owns the best appropriate

superiority before understanding the functional mechanisms during the progress of osseointegration. The high resolution SEM observation on argon ion beam polished cross section revealed more details to expound the different surface functions including promoting fibrous bone rooting by surface macroporous pores or micron-sized concavities and reinforcing bone and implant.

5. Conclusions

Argon ion beam polishing is a promising approach for preparing well-polished surfaces for characterizing the microstructure of newly formed bone and its interface with different implants after *in vivo* test by high resolution scanning electron microscope. The bone formed by two growth mechanisms, contact osteogenesis and distant osteogenesis, are verified visually. The newly formed bone from old bone and on implant surface can be distinguished. The increase of bone density and the bone structure change with time can also be observed directly. Besides, the correlated to the characters of the implant surfaces provide a better view of osseointegration at microscopic level. The functional mechanism of different implant surfaces during osseointegration are revealed; bone can grow into the surface pores of TiUnite® implants and micron-sized concavities of the SLM implant surface; and bone grows closely and tightly along the sand-blasted implant surface.

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