Bone response to surface modified titanium implants – studies on the tissue response after 1 year to machined and electropolished implants with different oxide thicknesses

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The bone formation around titanium implants with varied surface properties was investigated after 1 year in rabbits. Machined and electropolished samples with and without thick, anodically formed surface oxides were prepared, surface characterized and inserted in the cortical bone of rabbits. Scanning electron microscopy, scanning Auger electron spectroscopy and atomic force microscopy revealed marked differences in oxide thickness, surface topography and roughness, but no significant differences in surface chemical composition between the different groups of implants. Light microscopic morphology and morphometry showed that all implants were in contact with bone and had a large proportion of bone within the threads. There were no significant differences between the differently prepared implant groups. Our study shows that a high degree of bone contact and bone formation is achieved after 1 year with titanium implants which are modified with respect to oxide thickness and surface topography. There is no indication that a reduction of surface roughness, which in the initial phase decreases the rate of bone formation, had any influence on the amount of bone after 1 year in rabbit cortical bone.

1. Introduction

The importance of the surface properties of biomaterials for tissue responses has been identified and it has also influenced the basic and applied research within the area of medical devices. However, there is yet insufficient knowledge about which structural and chemical surface properties influence the biological responses. Further, there is also a need to understand how the material properties are transduced into the biological environment.

Major interest has been devoted to molecular and cellular reactions *in vitro*, with surfaces subjected to modifications and characterization [1–9]. Obviously, protein adsorption and migration, adhesion and various functional expressions of cells in contact with or in the vicinity of surfaces may be studied in much greater detail *in vitro*. However, in search for correlations between surface properties and biological responses there is also a need to evaluate, at different levels of resolution, the response of tissues under *in vivo* conditions. The objective and rationale of the present interdisciplinary research programme has been presented in more detail in an earlier report [10]. A systematic variation and characterization of surface properties is achieved either by selecting different, but related, materials, or by surface modification of a single material. Hitherto, our studies (performed in cortical bone of rabbits) have been focused on the properties of the titanium surface oxide, which has been pointed out as an important factor [11, 12] for the osseointegration process. Recent *in vitro* and *in vivo* studies provide strong indications that biological responses to titanium are influenced both by surface structure (roughness) and chemical composition [4, 13–21]. In most studies, however, the type of surface preparation and/or characterization methods used prevent any firm conclusions being drawn as to which surface properties were the determining factor for the observed differences in biological response. Studies on retrieved metal implants, including titanium, have

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indicated that the thickness of the surface oxide layer increases with time and that ions (calcium, phosphorus, sulfur) from the physiological environment are incorporated into the growing oxide [22].

Previous studies have shown that surface properties of titanium implants, such as topography and roughness, oxide thickness and microstructure, oxide composition, impurity levels, etc., vary considerably, depending on the type of surface preparation used (see [23] and references therein). These studies also underline the fact that an intentional change in surface roughness (submicrometre level) often also leads to (non-intentional) changes in the surface composition and in the oxide thickness. The surface properties of titanium can, however, be varied over a wide range in a more or less controlled and systematic manner, provided that proper preparation and characterization procedures are used.

Few studies have been carried out to investigate systematically the role of individual surface (oxide) properties of titanium on the biological response, because it is difficult to isolate one property. In the present work, we have investigated whether the oxide thickness and/or surface topography could influence the healing of bone adjacent titanium implants. In a previous study, we examined the early (1–6 week) formation of bone in the rabbit tibia around four differently modified titanium surfaces [24]. On the background of the results obtained it was of definite interest to evaluate the long-term bone response.

2. Materials and methods

2.1. Implant preparation

Implants were prepared in an identical manner as in our previous short-term study [24]. A total of 24 threaded implants (3.75 mm diameter, 4.0 mm length) were manufactured by machining of a commercially pure (99.7%) titanium rod (Permascand, Ljungaverk, Sweden). All samples were ultrasonically cleaned: trichlorethylene 10 min, acetone 10 min, and methanol 10 min of analytical grade purity. Four different groups of samples were prepared according to the procedures summarized in Table 1. Six of the machined implants served as control samples and the remaining 18 were modified using previously described electrochemical methods (electropolishing and/or anodic oxidation) [25–27].

Electropolishing, which acts as a controlled electrochemical dissolution of the surface [28], removed less than 100 μm of material from the surface. The electropolishing procedure was carried out in order to produce a smooth, shiny surface finish. The anodic oxidation (anodization) [29] procedure produced a vivid, greyish-purple coloration of the surface, due to light interference in the thick oxide that was formed.

All samples received a final ultrasonic cleaning step in ethanol (70%) for 3×10 min. Immediately after cleaning, the implants were put on a chemically pure titanium tray with a chemically pure titanium lid in separate compartments. The tray was put in a polymer sterilizing bag, sealed with sterilizing tape and steam sterilized in a conventional autoclave (120 °C for 45 min), prior to surgery.

2.2. Implant characterization

2.2.1. Surface elemental composition

From scanning Auger electron spectroscopy (AES) analysis, the surface elemental composition of two samples of each preparation type were obtained. Relative concentrations (at %) of elements within the probed volume (typically the outermost three to ten atomic layers) were calculated as the mean value from two or five points located in the threaded portion of each sample and after correction for sensitivity factors [30]. The oxide thickness was estimated from the depth profiles as the depth at which the oxygen signal had decreased to half of its maximum intensity. A more detailed description of the characterization of the surface elemental composition may be found elsewhere [24].

2.2.2. Surface topography and roughness

Scanning electron microscopy (SEM, Zeiss DSM 982 Gemini) was used to obtain an overall picture of the surface finish and topography of the samples. Scanning electron micrographs were taken at several randomly chosen areas on the implant surfaces.

A quantitative characterization of the surface topography and roughness was carried out by atomic force microscopy [31] (AFM, Nanoscope III, Digital Instruments, USA). One sample of each preparation type was analysed at ten randomly chosen areas ($1 \times 1\ \mu m^2$, 256×256 pixels) on the bottom of the implant. In a previous study, analysis at this location of the implant was found to give the same result as from the threaded part [10].

The surface roughness, R_{rms}, of each imaged area was determined using the computer software of the AFM instrument, and mean values were calculated for

TABLE I Results from AES and AFM investigations of screw-shaped titanium implants; oxide thickness, surface roughness, R_{rms}, and surface area enlargement, A_{diff}

Preparation	Oxide thickness (nm)	R_{rms} (nm) (S.D.)	A_{diff} (%) (S.D.)
Machined (control)	3–5	30.3 (19.8)	10.8 (7.6)
Machined + anodized	180–200	40.8 (14.7)	18.0 (8.2)
Electropolished	2–3	2.9 (2.9)	0.5 (0.4)
Electropolished + anodized	180–200	32.3 [2.7 (0.2) 116.7 (40.2)]	23.3 [0.6 (0.1) 88.0 (35.0)]

each type of surface. This parameter gives a measure of the roughness over a given area, in contrast to conventional stylus methods which measure along a line in a chosen direction. In addition, the AFM images were used to calculate the surface area enlargement, A_{diff}, which represents the enlargement in surface area (in per cent of the projected area) caused by surface roughness in the range from a few nanometres (4 nm $\approx$ resolution of the images) up to 1 μm (size of imaged area). The surface area enlargement was estimated from the sum of the area of all triangles formed by three adjacent pixels divided by the projected image area [32].

2.3. Animals and surgery

Six adult New Zealand White female rabbits, weighing 3–4 kg, were used. The experiment was approved by the local ethics committee. The animals were allowed to run free in a specially designed room with food and water *ad libitum*. The rabbits were anaesthetized by intramuscular (i.m.) injections of a combination of phentanyl and fluanizon (Hypnorm Vet.®, Janssen Farmaceutica, Denmark) at a dose of 1 mg/kg body weight (b.wt.) and intraperitoneal (i.p.) injections of diazepam (Apozepam®, Apothekarnes lab. A.S. Oslo, Norway) at a dose of 2.5 mg/kg b.wt. Local anaesthesia, lidocain (5% Xylocain®, Astra, Södertälje, Sweden) was applied in the skin and periosteum. Implantation was made bilaterally in the tibial bones. After incision through the skin and periosteum, a flap was raised to expose the bone area. A careful surgical technique was applied with generous irrigation with saline and low-speed drilling. After pre-threading, two implants were inserted 10 mm apart in each proximal metaphysis in a pre-determined order. Thus, each animal received one implant of each type, altogether four implants.

The animals were killed with an overdose of barbiturates intravenously (i.v.) and fixed by perfusion with 2.5% glutaraldehyde in 0.05 M sodium cacodylate, pH 7.4. The implants and surrounding tissue were removed *en bloc*, further immersed in glutaraldehyde overnight and then postfixed in 1% osmium tetroxide, for 2 h. After dehydration the undecalcified specimens were embedded in plastic, LR White® (The London Resin Co. Ltd, Hampshire, UK).

2.4. Morphology and morphometry

Ground sections of 10–15 μm thickness were prepared [33] and examined, using a Leitz Microvid equipment connected to a personal computer. Measurements were performed directly in the microscope. The contact ratio between the implant surface and bone tissue was calculated. Similarly, the proportion of bone tissue within the threads along the implant was calculated. The data are given as percentage bone in direct contact with the implant (referred to as bone contact) and percentage of the total area within the threads occupied by mineralized bone (referred to as bone area). All five consecutive threads (with numbers 1 and 2 located in the cortex) were evaluated. The mean value for each implant type was calculated and compared. In addition, the bone contact and bone area in the three best consecutive threads was evaluated.

2.5. Statistics

The Wilcoxon signed rank test was used.

3. Results

3.1. Implant surface characterization

A comprehensive description of the surface elemental composition, oxide thickness, topography, and roughness may be found in Larsson *et al.* [24].

3.1.1. Surface composition and oxide thickness

The AES analyses showed that all samples had a relatively similar surface composition, dominated by strong titanium, oxygen and carbon signals in the spectra, independent of preparation. The O/Ti (418 eV) peak height ratio was ~ 4 for the control samples and ~ 3.5 for the modified samples. The high O/Ti ratios are most likely due to excess oxygen bound to other elements than titanium, such as hydroxyl groups and oxygen in different organic groups [23, 24]. The shapes of the AES Ti_{LMV} peak indicated that the surface oxides had a TiO_2-like stoichiometry [23, 34, 35]. The control samples and those which had been electropolished had thin oxides (< 5 nm), with no significant difference between them. The two groups of anodized samples had thicker and similar oxide thicknesses (180–200 nm). The main surface contaminant was carbon (25–40 at %), most probably from adsorbed hydrocarbons originating from rinsing solvents, autoclaving and air exposure. The carbon contamination levels varied between the machined and electropolished samples. Trace amounts (less than a few per cent) of calcium, sulfur, phosphorus and silicon were detected on most sample types, while aluminium contamination (6%–17%) was occasionally found on some of the machined (control) samples.

The AES results are in good agreement with previous studies of titanium surfaces [23, 25–27, 34] and showed that the different surface preparations had produced the intended variations in oxide thickness and no major differences in chemical composition between the samples.

3.1.2. Surface topography and roughness

Scanning electron micrographs of the surface topography of the four different types of implants showed that the machined and the machined plus anodized surfaces had a relatively similar appearance, with the machining grooves in the 1–10 μm range as a main feature. The machined plus anodized surface also showed an additional, irregular surface roughness on the ~ 1 μm level and smaller, which was superimposed over the grooves. The electropolished

surface appeared very smooth in the SEM. The electropolished plus anodized surface had a heterogeneous surface topography consisting of mainly ($\approx$ 75%) smooth areas and some rough regions of size 10–100 μm. Traces of machining grooves could be observed on neither of the two groups of surfaces that had been electropolished.

In the AFM, the machined sample showed machining grooves of 1 μm width and lower. At the submicrometre level, the surface also showed clear corrugations on the 0.1 μm scale and smaller, which appeared to occur preferentially in a direction normal to the machining direction. On the machined plus anodized sample, the machining grooves were less distinct on the submicrometre level which was dominated by relatively smooth elevations and recesses of sizes of a few tenths of a micrometre, but with no preferential direction. This topography probably represents that of the ($\sim$ 0.2 μm thick, see below) anodic oxide which had been formed over the original machined surface.

The AFM images confirmed that the electropolished surface was extremely smooth, with occasional pits and grain boundaries (or dislocations) as the only features observable on the 1 μm scale. On a submicrometre scale, the surface appeared granular, with an average feature size of approximately 40 nm in diameter and 2–5 nm in height. This topography is similar to that previously observed on electropolished titanium surfaces [10, 24, 36–38]. The two types of areas (smooth and rough) on the electropolished plus anodized surface, observed both by LM and SEM, were quite different also in the AFM images. The smooth areas consisted of flat regions, frequently containing what appeared to be pores or pits of sizes typically 400 nm in diameter surrounded by elevated edges. The rough areas consisted of deep pits of sizes of a few micrometres and with sharp ridges. At the submicrometre scale, the topography of the areas in between the pits was very similar to that of the machined plus anodized surface.

The compiled data on the surface roughness and surface area measurements by AFM are given in Table I. In brief, the electropolished implants had the smoothest surface ($R_{rms} = 2.9$ nm), and a negligible surface area enlargement (0.5%). The smooth areas on the electropolished plus anodized samples were similar to the electropolished surface, while the rough areas had an $R_{rms} \approx 117$ nm and 88% surface area enlargement. The machined and machined plus anodized surfaces had similar and intermediate surface roughnesses ($R_{rms} \approx 30$ and 40 nm, respectively), and the enlargement in surface area was slightly larger for the latter surface (11% and 18%, respectively).

The SEM and AFM results showed that the different preparations had produced surfaces with a variety of different surface topographies and roughnesses on the scale of less than 0.1 μm and up to at least $\sim$ 10 μm.

3.2. Morphology and morphometry

The implantation site, the metaphysis of the tibia, consists of mainly cortical bone. The one or two most proximal threads of the implant are located within the cortex. The remaining part of the implant protrudes into the marrow cavity without contacting the endosteal surface of the opposite cortex. As previously described [10, 24, 39–41], the formation of new bone around titanium implants takes place around the cortical (proximal one or two threads) as well as the intramedullar (distal three to five threads) portion of the implants.

The results of the morphometric evaluation of the relative bone area and bone in contact with the implant are shown in Fig. 1 and Table II. No differences between the machined and electropolished groups were found. Densely mineralized lamellar bone surrounded the implants and filled all the threads (Fig. 2a, b). In the LM, a close contact between the

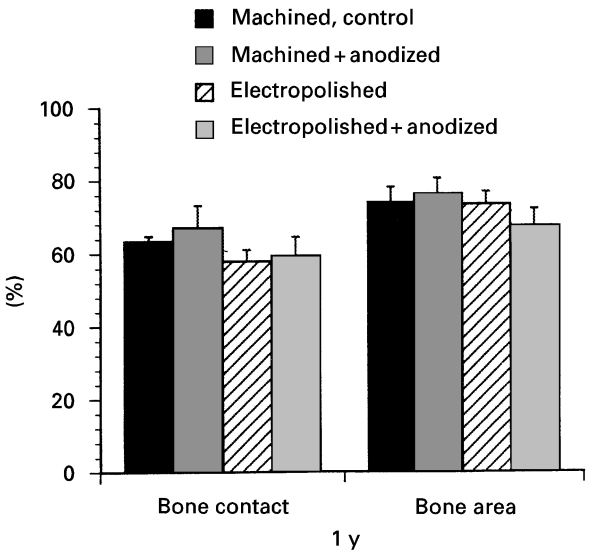

Figure 1 Morphometry: total bone contact (%) (mean + S.E.) and total bone area (%) (mean + S.E.). Machined (control), 3–5 nm; machined + anodized, 180–200 nm; electropolished, 2–3 nm; electropolished + anodized, 180–200 nm.

TABLE II Histomorphometric evaluation. Bone area (%) and bone contact (%). Values for all threads are compared with the three best consecutive threads

Preparation	All threads, bone area (%)	Three best threads, bone area (%)	All threads, bone area (%)	Three best threads, bone contact (%)
Machined, control	74.0	89.5	63.4	75.6
Machined + anodized	76.3	90.5	67.1	75.5
Electropolished	73.6	89.9	58.0	74.1
Electropolished + anodized	67.7	89.5	59.4	68.6

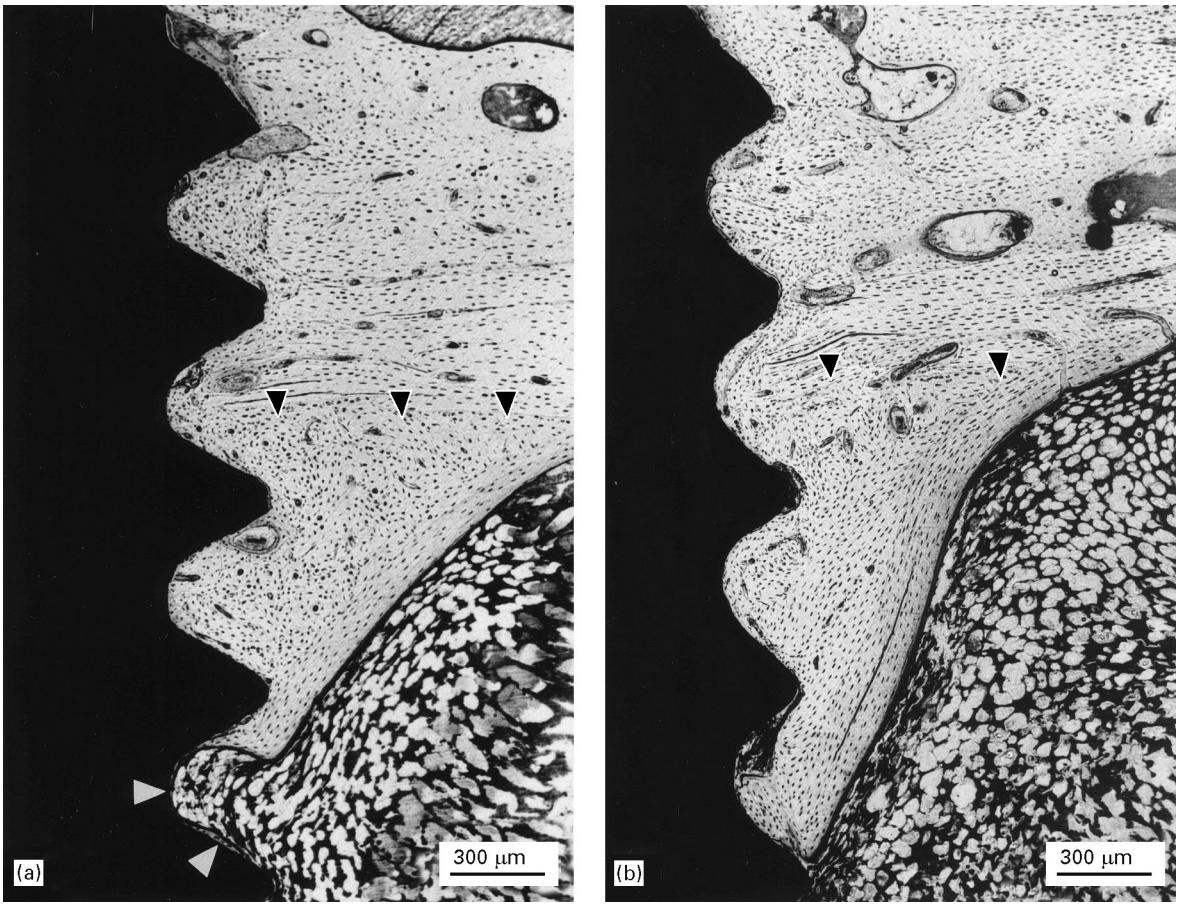

Figure 2 Light micrographs showing a collar of mineralized bone enclosing the implant. Bone is in contact with the implant surface irrespective of surface modification after 1 year. A thin lining of mineralized bone is in direct contact with the implant surface without a fully filled thread in (a) (white arrow-heads). The border between the old cortex and the newly formed bone is hardly detectable (black arrow-heads); (a) machined + anodized implant, and (b) electropolished implant.

mineralized bone and the implant surface was infrequently seen in all threads, irrespective of surface modification (Fig. 3a–d). The flat-bottomed portion of the implant and occasionally the adjacent bottom threads, were only in contact with the marrow tissue.

4. Discussion

In the present study, the cortical bone response was evaluated around non-functionally loaded titanium implants after a relatively long healing period (1 year post-implantation) in rabbits. The implants were subjected to surface modifications using electropolishing and anodization. SEM, AES and AFM revealed marked differences in oxide thickness, surface topography and roughness, but no major differences in chemical composition between the four implant groups. The major biological observation in the present study was that the four implant types; machined, machined and anodized, electropolished, and electropolished and anodized implants, were all surrounded by mature, lamellar bone in contact (at LM level) with the implant surface. Further, our morphometric evaluation (Table II and Fig. 1) showed that all implant types had a high degree of bone-to-implant contact and a high proportion of bone within the threads, but no significant differences between the groups could be observed.

Comparisons between results from different studies are often difficult because the animal species, experimental model, observation period, methods of evaluation, implant design and surface properties often vary. In the literature there are few published reports on 1 year follow-up periods. In a 1 year study by Wennerberg *et al.* [42] using the same animal species and experimental model, rough (Al_2O_3)-blasted titanium implants were found to have a higher amount of bone in contact with the surface and higher removal torques in comparison with relatively smoother, machined, titanium implants. Interestingly, our morphometric data (total values and the three best consecutive threads) on the bone contact and bone area within the threads, are equal to or higher than the values given for both rough ($S_a = 1.16$ and 1.94 μm) and smooth ($S_a = 0.96$) implants [42]. One possible explanation for the high values in the present study could be that the surface microtopography on the submicrometre level may be as important for the long-term bone response as the topography on the 1–10 μm level. The AFM method as used in this study, for characterizing surface topography takes into account roughness on an area of $1 \times 1\ \mu m^2$ at

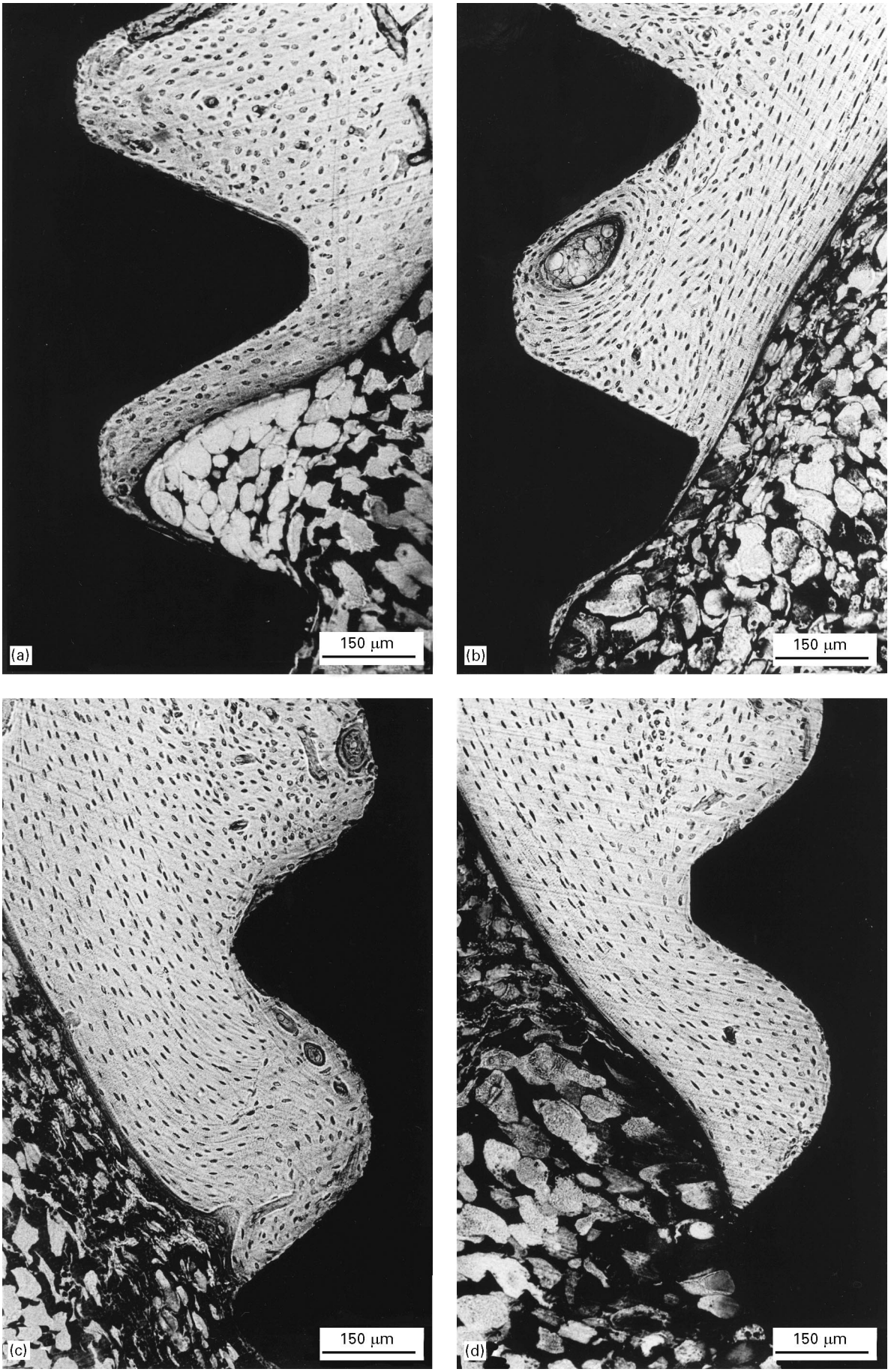

Figure 3 Survery light micrographs of the bone response to the different surfaces: (a) machined, control; (b) machned + anodized; (c) electropolished + anodized.

a lateral resolution of a few nanometres. Conventional R_a values, only including roughness on a lateral scale and resolution larger than ~ 1 μm, for machined titanium surfaces has been reported to be typically 0.1–0.2 or 0.5 μm, as measured by stylus along a line [43] or optical techniques [44], respectively. An R_a value of 0.5 μm has been measured for machined titanium surfaces prepared according to an identical procedure as in the present study (unpublished data). Thus, all surfaces used in this study are still relatively smooth compared to, for example, sandblasted or plasma-sprayed surfaces.

In our previous study [24] on the early bone response to identical implants, the electropolished implants had less bone within the threads and a lower degree of bone-to-metal contact than the other three groups. These short-term data indicate that the surface characteristics of the non-anodized electropolished implants (combination of a relatively thin layer of surface oxide and a relatively low degree of roughness) may represent unfavourable properties for the initial bone growth. It is therefore interesting that the differences in oxide thickness and surface roughness do not lead to any considerable difference in the morphological results 1 year after non-functional loading. We have no clear explanation for these observations. Observations [10, 24, 44] indicate that thicker surface oxide and higher roughness presents particularly favourable conditions during early time periods (1–7 weeks). This is in agreement with observations made by Hazan *et al.* [13] who showed that heat treatment of titanium alloy implants, and thereby increased oxide thicknesses, leads to higher removal torques and a higher degree of calcification of the bone around implants inserted in the medullary in rats.

One may speculate about several different possible explanations for the observed long-term results in the present study. One could be that the electropolished non-anodized surface has acquired a thicker oxide and thereby an increased roughness during the 1 year implantation period. The hypothesis that the titanium surface oxide may grow under *in vivo* conditions has been tested in one previous study. In a human retrieval study, the thickness of the surface oxide layer had increased with time and ions (calcium, phosphorus) from the physiological environment had become incorporated into the surface oxide [22]. Obviously, it is of interest to examine if such an increase in oxide thickness and roughness had occurred also with the present implants, and, in turn, to evaluate if oxide thickness variations *in vivo* are associated with osseointegration or failure of osseointegration.

A second explanation could be that the rate of bone formation around these implants is influenced by ion release rates. Titanium ions have previously been shown to have an inhibitory effect on calcification *in vitro* [45]. Ion release rates *in vitro* from titanium materials decay with time due to self passivation [46, 47]. The absolute ion release rates are low (p.p.m. levels per cm^2 sample area after several months in test solutions) but can be expected to depend on a number of oxide properties, such as thickness, morphology, crystallinity and defect density, and thus on the surface preparation. To our knowledge, ion release rates have not been measured for this particular type of modified titanium surfaces, and the effects of low levels of ions on cellular behaviour is not known. Therefore, we cannot exclude that the slower formation of bone around the electropolished implants at short time periods is, at least partially, due to higher ion release rates from these surfaces.

The observations from our previous and the present study should also be discussed in relation to observations of cellular behaviour at modified titanium surfaces. A literature survey indicates that the response of cells to variations in culture substrate topography varies between different cell types, including macrophages [48, 49], fibroblasts [50], periodontal cells [51], epithelial cells [16, 17], osteoblasts [4, 8] and chondrocytes [9]. Osteoblasts have an initial, greater attachment to rough, sandblasted titanium surfaces with irregular morphology [4], but according to these authors, average roughness, R_a, values do not predict cell attachment and spreading *in vitro*. Further, Martin *et al.* [8], have demonstrated that osteoblast-like cell proliferation, differentiation and matrix production are altered by surface roughness. Interestingly, it appears as if cells at different stages of differentiation *in vitro* respond differently to the same surface [9, 52]. Thus, in relation to our previous *in vivo* findings [10, 24] and the present results it is possible that the outcome of the encounter between the artificial surface and bone *in vivo* could be different at early and late time periods, depending on a change in the interface of the type of cells and their maturity stage. Moreover, it is likely that artificial materials are recognized in different ways by cells depending on the specific macromolecules adsorbed to the implant surface and their conformation state. Because the surface area/roughness might influence the amount of adsorbed proteins it is possible that the variations of surface topography could also influence cells indirectly.

The concept of osseintegration was developed by Brånemark *et al.* [53] at the end of the 1960s, and has resulted in a predictable long-term success of osseointegrated oral implants. A large number of working definitions of this term has been provided [54–58] including definitions based on morphological criteria. In a study of retrieved, clinically stable titanium implants (manufactured in an identical manner and with the same surface characteristics as the machined implants in the present study) a high degree of bone-to-implant contact and bone area within threads were detected [59]. In contrast, clinically failed osseointegrated implants, clinically manifested by a peri-implant radiolucency and mobility, were surrounded by a fibrous tissue with a large number of cells with CD62 positive immunoreactivity (macrophages) [60]. From a clinical perspective, therefore, it appears crucial to establish an early implant stability and to maintain osseointegration. Although the bone around non-functionally loaded implants was evaluated, the results from the present study indicate that titanium implants with similar chemical composition but large variations in surface oxide thicknesses and micro-topography may become equally well osseointegrated

under experimental, non-loaded conditions after 1 year. In view of the advantage to reach a high degree of stable fixation as early as possible while still maintaining osseointegration in the long-term, an evaluation of the clinical performance of machined and electropolished titanium implants is well motivated.

5. Conclusion

Regardless of surface modification, a high degree of bone–implant contact was found for all titanium implants studied. The electropolished implants, which had a smooth surface with a thin oxide, did not result in a significantly lower bone growth around the implants than the other groups. Anodic oxidation of the electropolished surfaces, which produced areas of increased roughness and a thicker surface oxide, did not influence the surrounding tissue in a negative way after 1 year. Increasing the oxide thickness of rough machined implants had no significant effect on the bone response. The results show that the surface topography on the submicrometre scale and the oxide thickness do not affect the bone response to titanium after longer periods.

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