Tissue response to hafnium

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The aim of the present experimental study was to evaluate the tissue response to hafnium (Hf) a reactive metal closely related to titanium (Ti) and zirconium (Zr). Hf has not been previously evaluated as implant material in a biologic environment. In a first experiment, 21 machined Hf non-threaded implants (test) and 21 similar Ti implants (control) were inserted in the abdominal wall of 21 rats. Animals were sacrificed after 8 days (6 rats), 6 (7 rats) and 12 weeks (8 rats). In a second experiment, 18 rabbits received 18 Hf and 18 Ti threaded implants in their tibiae, one implant in each tibia. The rabbits were sacrificed after 6, 12 and 24 weeks (6 animals/time interval). The bulk metal of the abdominal wall implants, embedded together with the surrounding tissue, was electrolytically dissolved and semithin (1 μm) sections of the intact tissue–implant interface were evaluated by light microscopy (morphometry). Bone-implant contact and bone area within threads were evaluated in ground sections. In soft tissues, a fluid space containing predominantly monocytes/macrophages surrounded the abdominal implants at 8 days. At 6 and 12 weeks, a fibrous capsule, consisting of layers of macrophages and fibroblasts, surrounded the implants. Macrophages, including multinuclear giant cells, always formed the innermost layer in contact with the implant surface. No quantitative or qualitative difference in the tissue organization was detected between Ti and Hf implants. In rabbits, 6 weeks after insertion, the proximal two threads located within the cortical bone were filled with bone in contact with Hf and Ti. The distal threads contained bone marrow. After 12 and 24 weeks, mature bone was present in the proximal 3–4 implant threads. No statistically significant difference was found between Hf and Ti implants at any time periods. It is concluded that Hf is an interesting metal for biomedical applications in bone and soft tissue.

1. Introduction

The material properties of implants used in the human body can play a significant role in the clinical outcome of reconstructive therapies. Ti, due to its biocompatibility, high corrosion resistance and low cost, is one of the most used and documented biomaterials with excellent clinical results in orthopedic and cranio-maxillo-facial applications [1]. Ti may also serve as a valuable reference material and as substrate for further surface modifications. There are other metals with chemical characteristics similar to Ti: Hf and Zr. All these metals have similar outer shell valence electron structures and are found in the same group (IVB) of the periodic table of elements. These transition elements are also called ''reactive'' metals since they are very reactive to oxygen. In fact, once exposed to air, a thin, but very stable oxide layer is immediately formed on their surfaces. The surface oxides are considered as ceramics and not pure metals [2]. The chemical stability of the oxide layer explains the excellent corrosion resistance of these metals in most environments. Hf and Zr are considered to be ''refractory metals'', having a melting point $> 1750\,^{\circ}C$ (Hf 2222 °C and Zr 1852 °C). Although Ti has a melting point of 1668 °C, it still may be classified as a refractory metal [3].

Zr is not yet widely used as a biomaterial but has been shown to possess interesting properties in tissues under experimental conditions [4–6]. Nothing is actually known about the tissue response to Hf as implant material. Hf (atomic number 72) is a silvery ductile metal. It was discovered in 1923 in Copenhagen and was named after ''Hafnia'', the Latin name for Copenhagen. It is found in all Zr-containing minerals, often in small proportions (1–5%), and has to be separated from Zr. Of all elements, Hf and Zr are the most difficult to separate. Although their chemistry is almost identical, the density of Hf is about double of that of Zr. Very pure Hf has been produced with Zr as the major impurity. Hf properties are considerably influenced by the impurities of Zr present. In contrast to Ti, Hf is one of the less abundant element in the Earth's crust. In industry, Hf is generally derived from certain beach sands. Because of its high corrosion

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resistance, good mechanical properties and good absorption of thermal neutrons (almost 6000 times that of Zr), the most important application of Hf is in nuclear reactor control rods of nuclear submarines [3].

The aim of the present study was to evaluate the biologic response to Hf, using Ti as control material, in both soft tissue (rat model) and cortical bone (rabbit model).

2. Materials and methods

2.1. Implants

Implants were manufactured by machining rods 5 mm in diameter of Hf (1.24% Zr content) and Ti (grade 1, 99.7% Ti) obtained from Edstraco, Stockholm, Sweden. The Hf and Ti implants, used in soft tissues, consisted of a circular plate (thickness 0.5 mm; diameter 4 mm) connected to a cylindrical hollow internal threaded rod (length 3 mm; diameter 2 mm) as previously described [7]. The machined Hf and Ti implants, inserted in bone, were threaded (length 4.0 mm, diameter 3.75 mm). The top of the implant had a slit to fit a screw-driver during insertion.

Before implantation, the implants were cleaned ultrasonically in trichloroethylene (15 min), in acetone (15 min), in absolute ethanol (3 × 10 min) and sterilized by autoclaving.

The implant surface morphology was examined by scanning electron microscopy using a Jeol JSM T-300.

2.2. Animals and surgical procedures

The experiments were approved by the Gothenburg Local Ethical Committee. Surgery was performed under sterile conditions.

2.2.1. Soft tissue (rats)

Twenty-one adult Sprague-Dawley male rats (Alab, Södertälje, Sweden) weighing 250–300 g, fed on a standard pellet diet and water *ad libitum* were used. Six animals were included in the 8-day, 7 in the 6-week period and 8 in the 12-week observation period. The rats were anesthetized with an intraperitoneal injection of 2 : 1 : 1 solution of sodium pentobarbital (60 mg/ml, Apoteksbolaget, Göteborg, Sweden), diazepam (5 mg/ml, Apozepam, Apothekarnes Laboratorium AS, Norway) and 0.9% saline. Hf and Ti implants were inserted in the abdominal wall, using a procedure previously described [7]. Each rat received two implants, one of each material, placed 10 mm apart immediately right to the linea alba. The cylindrical portion penetrated through the peritoneal membrane into the abdominal cavity, while the circular plate was located inside the abdominal wall between and in contact with the peritoneum and the *rectus abdominis* muscle. The muscle fascia and the skin were closed separately with resorbable sutures.

2.2.2. Bone (rabbits)

Eighteen adult New Zealand white rabbits of both sexes (Alab, Södertälje, Sweden), weighing 4–5 kg and fed *ad libitum* were used. Six animals were included in each observation period (6, 12 and 24 weeks). Animals were anesthetized by intramuscular injections of fluanizole (Hypnorm®, Janssen, Brussels, Belgium; 0.7 mg/kg body weight) and intraperitoneal injections of diazepam (Stesolid®, Dumex, Copenhagen, Denmark; 1.5 mg/kg body weight). Additional fluanizole was given every 30 min, during surgery.

Each tibial metaphysis was exposed by a skin incision and a periosteal flap was raised. A hole (1.8 mm in diameter) was drilled with a high speed hand-piece (2500 r.p.m.) under profuse saline irrigation. The hole was then enlarged to 2.0 mm and finally to 3.0 mm. After tapping with a low speed hand-piece (16 r.p.m.), an implant was inserted using a screw-driver. The implant was positioned with the upper portion in level with the outer surface of the cortical bone. Two implants (1 Hf and 1 Ti) were installed in each rabbit; one in each tibia. The periosteum and fascia were sutured with resorbable Vicryl® 5-0 suture and the skin with silk 3-0 suture. During 3 days postoperatively, the animals were given bensylpenicillin (Intencillin, Leo, Helsingborg, Sweden, 2.250.000 IE/5 ml, 0.1 ml/kg body weight) and analgetics (buprenorphine, Temgesic®, Reckitt and Coleman, USA, 0.05 mg/kg body weight) intramuscularly, once a day.

2.3. Tissue preparation

2.3.1. Soft tissue (rats)

Rats were anesthetized and fixed by perfusion via the left heart ventricle with 2.5% glutaraldehyde in 0.05 M sodium cacodylate, pH 7.4. The implants were excised together with the surrounding abdominal wall. The peritoneal surface with the implant cylindrical portion was photographed in a stereomicroscope in order to document the amount of tissue covering the cylinder. The implants with their surrounding tissue were left in glutaraldehyde overnight and then postfixed in 2% OsO_4 for 1 h. The samples were then dehydrated in ethanol and finally embedded in epoxy resin (Agar 100, Agar Aids, Stansted, Essex, England).

The bulk metal of the embedded specimens was removed by an electrochemical dissolution technique [8]. With this procedure only the thin surface oxide layer is left allowing the sectioning of an intact implant–tissue interface.

Light microscopic (LM) sections (1 μm thick) were cut on an ultramicrotome using glass knives and stained with Azur II and 0.5% methylene blue in 1% disodium tetraborate.

2.3.2. Bone (rabbits)

Animals were anesthetized by an intravenous overdose of pentobarbital (Mebumal®, ACO Läkemedel AB, Solna, Sweden) and fixed by perfusion with 2.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4 via the left heart ventricle for 5 min. The implants and surrounding tissues were removed *en bloc*, immersed in glutaraldehyde for 24 h and postfixed in 2% osmium tetroxide for 1 h. After dehydration in a graded series of ethanol, the specimens were embedded in plastic resin (LR White, The London Resin Co. Ltd., Hampshire, England) and divided in two

halves by sawing longitudinally through the implant (Exakt Apparatebau, Norderstedt, Germany). One half of each specimen was used to prepare a 10–20 μm thick section by grinding [9] and stained with 1% toluidine blue to be analyzed by LM.

2.4. Morphometry

2.4.1. Soft tissue (rats)

Semithin sections were analyzed as described elsewhere [10] using a Nikon Microphot-FXA light microscope with a 40× objective and a 10× eyepiece fitted with a square grid. LM morphometry of one section for each implant was performed on the tissue located at the surface of the plate portion facing the abdominal cavity, essentially following previously described procedures [11]. A dense line, representing the metal oxide surface, remaining after the electrochemical removal of the bulk metal (implant surface) was present in the sections. The width of the space, present after 8 days, between this line and the tissue was determined (fluid space). For each implant retrieved after 6 and 12 weeks, the width of the organized connective tissue (fibrous capsule) was measured at 5 predetermined points and expressed as a mean value. The evaluation of cell types, number and distribution was performed in 5 different areas along the interface. The first area was located 200 μm from the lateral border of the implant and the remaining 4 areas were located consecutively, separated by a distance of 120 μm, along the interface. In each area the tissue facing the implant was divided into 4 zones, each 20 μm in depth and 80 μm in width, with zone 1 located adjacent to the fluid space (8 days) or implant surface (6 and 12 weeks). In each zone the number of monocytes/macrophages and fibroblasts was determined. The number of other cell types (altogether about 10%), including nonidentified cells was also determined (data not presented).

The mean values for each zone obtained in the 5 areas in each section were calculated. The mean values for each group of rats were calculated on available retrieved specimens (8 days: Hf = 6, Ti = 5 (one specimen was lost), 6 weeks: Hf = 7, Ti = 5 (2 implants were lost) and 12 weeks: Hf = 8, Ti = 8). The data was statistically evaluated using Fisher's test for pair comparison (Bradley).

2.4.2. Bone (rabbits)

Qualitative light microscopic observations were made in a Nikon Microphot-FXA microscope. Morphometrical measurements were performed in Leitz Metallux 2 microscope equipped with a Microvid device connected to an IBM XT computer. The morphometrical analysis consisted of (1) determination of the degree of bone–implant contact, expressed as % bone contact, and (2) determination of the amount of bone within the implant threads, expressed as % bone area. The data was statistically evaluated using Fisher's test for pair comparison (Bradley).

3. Results

3.1. Surface topography

In the scanning electron microscope, the surfaces of Hf and Ti implants (Fig. 1) appeared to be qualitatively similar and were characterized by typical, parallel machining ridges and grooves which were 2–5 μm in width. Elevations and pits were superimposed on this pattern.

3.2. Tissue organization and morphometry

3.2.1. Soft tissue (rats)

No evidence of tissue overgrowth was detected on the intraperitoneal rod portion of any implant material (Fig 2). After 8 days, a fluid space was always observed between the plate portion of the implant surface, marked by a thin dark line (remaining surface oxide) and the surrounding tissue in sections of implant–tissue *blocs* (Fig. 3(a)). The fluid space contained scattered inflammatory cells, predominantly monocytes/macrophages and, in some specimens, also scattered erythrocytes. Very few cells adhered to the surface of the two types of implants. The width of the fluid space varied greatly within the same section as well as between sections of different specimens. The mean width of the fluid space around Hf implants was 23.8 ± 8.8 μm (mean ± standard error of the mean) and for Ti implants 59.2 ± 20.3 μm. Due to the insufficient number of available specimens no statistical conclusion could be drawn.

After 8 days, the surrounding tissue did not show any distinct organization, making the measurements of the capsule width uncertain. No difference in the tissue organization around the two types of implants was apparent. After 6 and 12 weeks, the fluid space had disappeared and a distinct fibrous capsule had been formed (Fig. 3(b) and (c)). After 6 weeks the capsule thickness around Hf was 106.2 ± 11.2 μm and around Ti 170.5 ± 19.9 μm (Fig. 4). The corresponding values after 12 weeks were 104.7 ± 17.6 μm around Hf and 111.3 ± 17.0 μm around Ti implants (no statistical difference at any time interval).

The innermost portion of the capsule consisted of layers of macrophages and fibroblasts. Macrophages were the most common cells detected around both implant materials at all 3 observation intervals. The highest concentration of macrophages was found in zone 1, closest to the implant surface (Fig. 5). The numbers of macrophages (mean ± SEM/1600 μm^2) after 8 days were: Hf = 5.50 ± 0.7, Ti = 6.6 ± 0.9; after 6 weeks: Hf = 8.4 ± 0.4, Ti = 9.1 ± 0.6, and after 12 weeks: Hf = 5.70 ± 0.6, Ti = 4.7 ± 0.25 (Fig. 5).

In contrast, an even distribution of fibroblasts was seen within the capsule. Focal accumulations of lymphocytes were also present around both implant materials, mainly in relation to blood vessels in zones 2 and 3. These cells were observed after 8 days around both materials. However, in comparison to macrophages and fibroblasts, the number of lymphocytes was low. There were no significant differences between machined Hf and Ti implants with regard to the presence and distribution of macrophages and fibroblasts at any time interval.

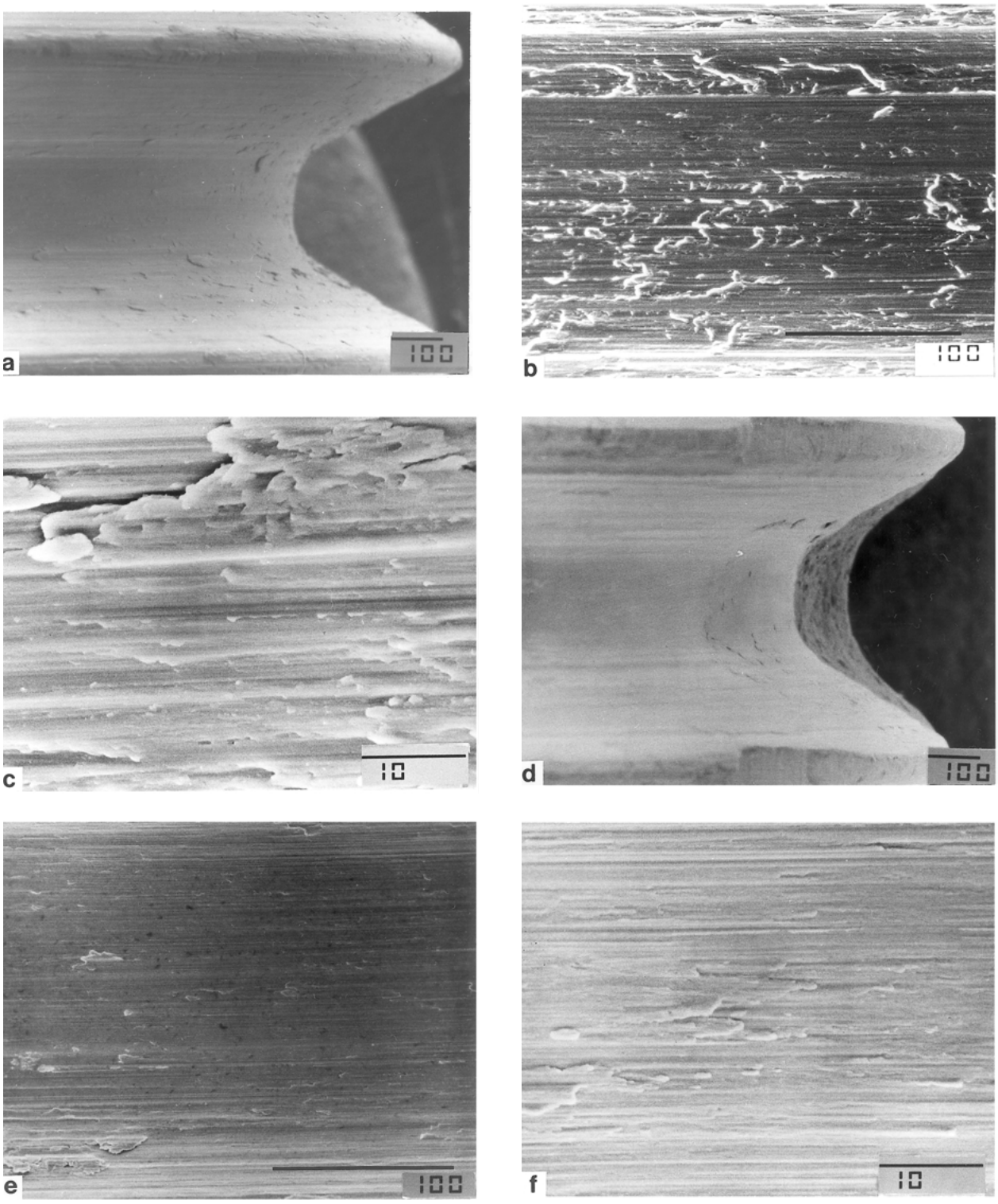

Figure 1 Scanning electron migrographs of implants used in bone. (a) Ti; Bar = 100 μm. (b) Ti. The implant surface displays grooves and ridges as well as protrusions. Bar = 100 μm. (c) Ti; Bar = 10 μm. (d) Hf; Bar = 100 μm. (e) Hf. Similarly to that observed for Ti, the surface exhibits grooves and ridges and small protrusions. Bar = 100 μm. (f) Hf; Bar = 10 μm.

3.2.2. Bone tissue

Light microscopy of the bone adjacent to Hf and Ti implants, 6 weeks after insertion, showed that the first two threads located within the cortical bone were filled with bone. The newly formed bone could be distinguished from the old cortical part. Bone trabeculae were observed extending from the endosteum as well as from the cut bone surface towards the implants. In general, the distal threads (#4 and #5) contained bone marrow tissue. After 12 and 24 weeks, the biological response still seemed to be the same for both materials. The tissue consisted mainly of well-organized and mature bone surrounding the implants at their proximal threads (#1–3) (Fig. 6).

After 6 weeks, morphometry of the bone around Hf specimens showed that both the bone area (mean $\pm$ standard error of the mean) and the degree of bone contact (mean $\pm$ standard error of the mean) in the proximal threads was similar to that observed around Ti specimens. The mean percentage of total bone area within threads of Hf implants was 36 $\pm$ 13.5 (41 $\pm$ 11 for Ti) and the mean percentage of the total bone-implant

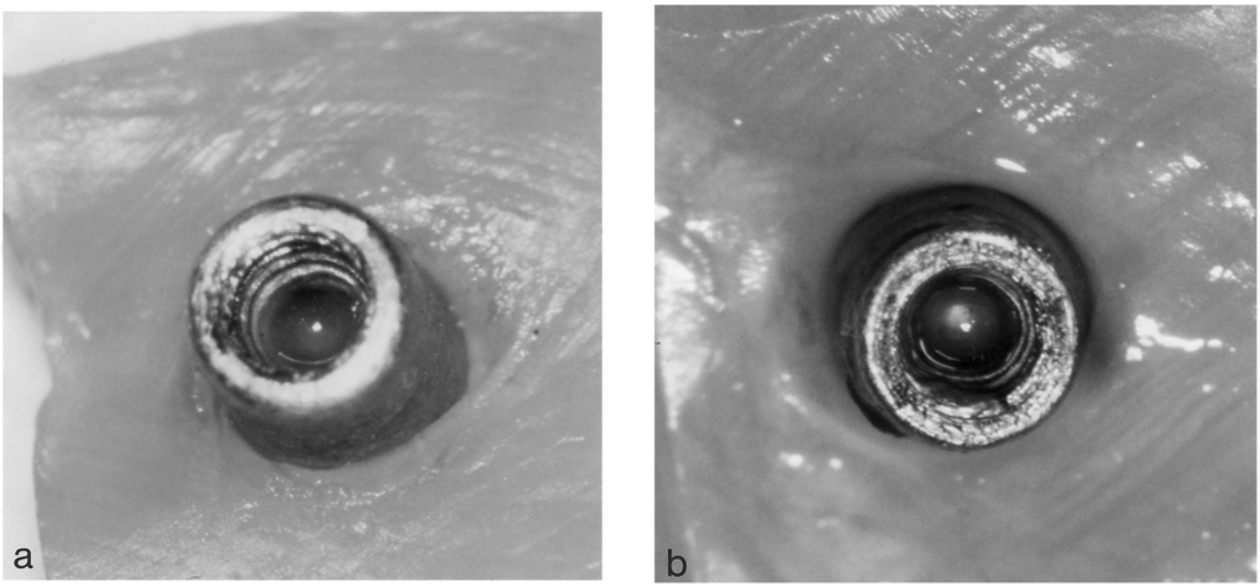

Figure 2 Photographs of the implants inserted in the abdominal wall of rats. The implants were removed after 6 weeks; (a) Ti implant, (b) Hf implant. The intraperitoneal part of the implants can be observed. The implants are free of tissue overgrowth. No macroscopic signs of inflammation can be detected in the surrounding peritoneal membranes. The hole in the cylindrical portion is used to mount the implant to the electropolishing equipment.

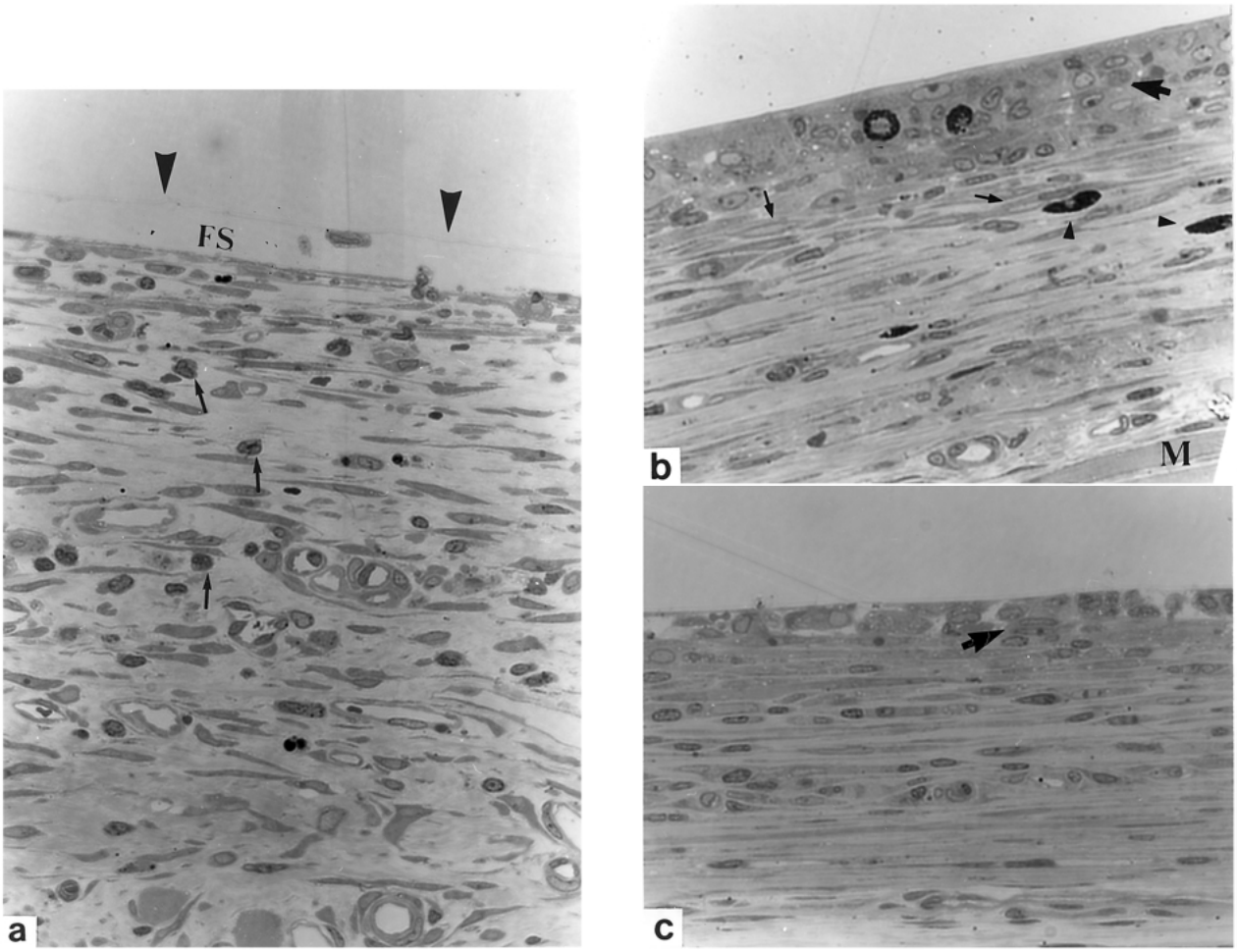

Figure 3 Light micrographs of tissues adjacent to the electrolytically removed Ti and Hf implants. (a) Hf, 8 days. The fluid space (FS), which separates the dense line (arrow-heads) representing the oxide layer after electropolishing from the tissue containing inflammatory cells. Macrophages (arrows) are scattered in the tissue. Numerous blood vessels are located in the reorganizing tissue. Magnification × 200. (b) Ti, 6 weeks. Elongated fibroblasts (small arrows) are located in the dense fibrous capsule between the implant surface and the surrounding muscle (M). Macrophages and some multinuclear cells (big arrow) are located close to the implant surface. Several mast cells (arrow-heads) are present. Blood vessels are located in the outer part of the capsule. Magnification × 150. (c) Hf, 6 weeks. A thin fluid space is present at the implant surface. Several macrophages (arrow) occupy the space, some of which are adherent either to the implant surface or to the tissue. Magnification × 250.

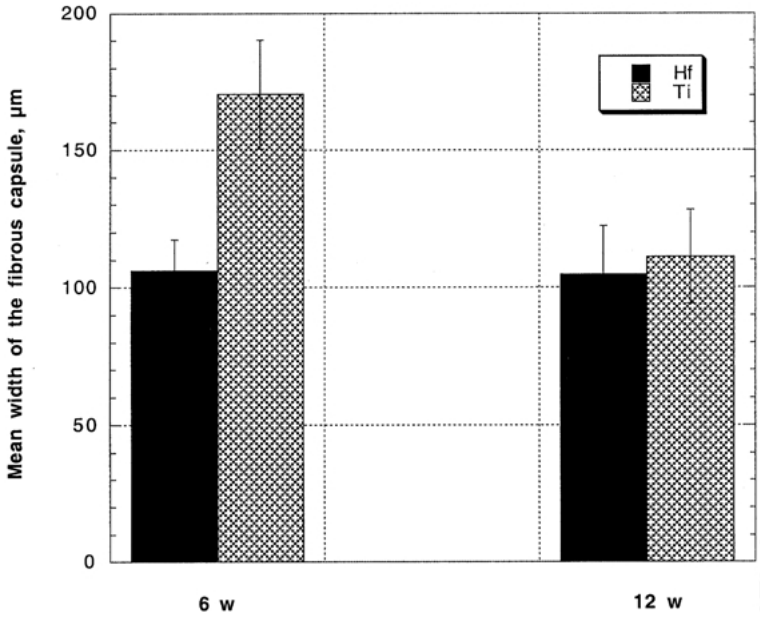

Figure 4 Histomorphometry/soft tissue. Thickness of the fibrous capsule around Hf and Ti implants after 6 and 12 weeks, respectively.

contact was 17.5 ± 5.8 (15 ± 4.5 for Ti) (Fig. 7). These differences were not statistically significant.

After 12 weeks, the mean total bone area within threads around Hf was 24% (35% for Ti). The total bone-implant contact had a mean of 7.3 ± 3 for Hf (13 ± 4.5 for Ti) (Fig. 7). Again, these differences were not significant statistically.

After 24 weeks the mean value for the total bone area around Hf was 34.5 ± 14.5 (45 ± 14 for Ti) and the mean total bone–implant contact for Hf was 18 ± 5.5 while Ti had 27 ± 7.3 (Fig. 7). These findings were not statistically different.

4. Discussion

The early *in vivo* response to implants to a major extent involves inflammatory and repair processes. The rela-

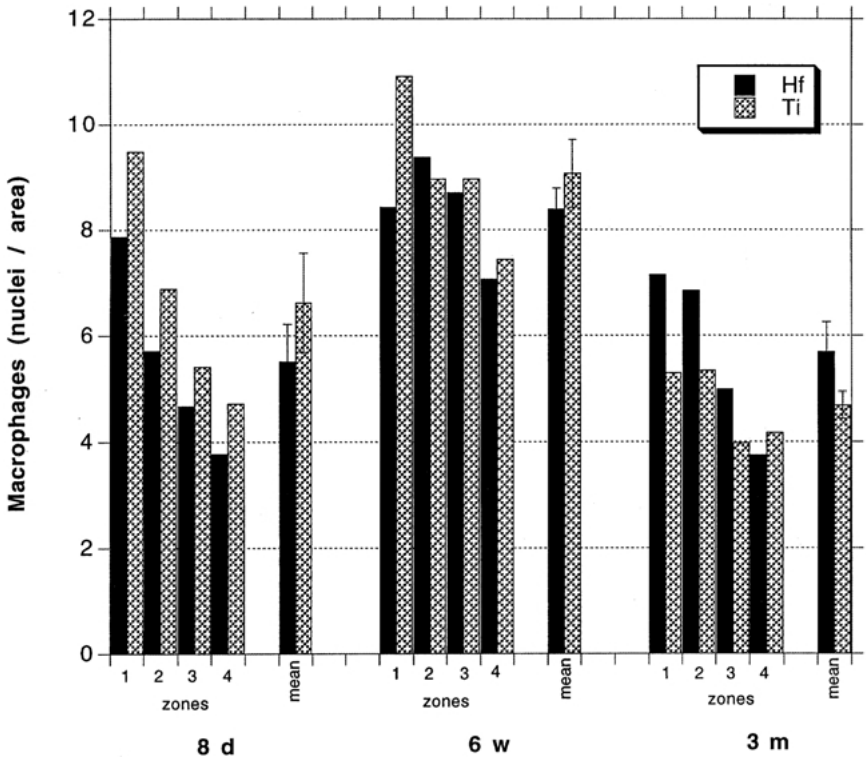

Figure 5 Histomorphometry/soft tissue. Distribution of macrophages in the tissue around the implants (for details see Materials and methods). The number of macrophages (nuclar profiles) in each zone (1600 µm^2) as well as the mean number (± SEM)/1600 µm^2 after 8 days, 6 weeks and 3 months are shown.

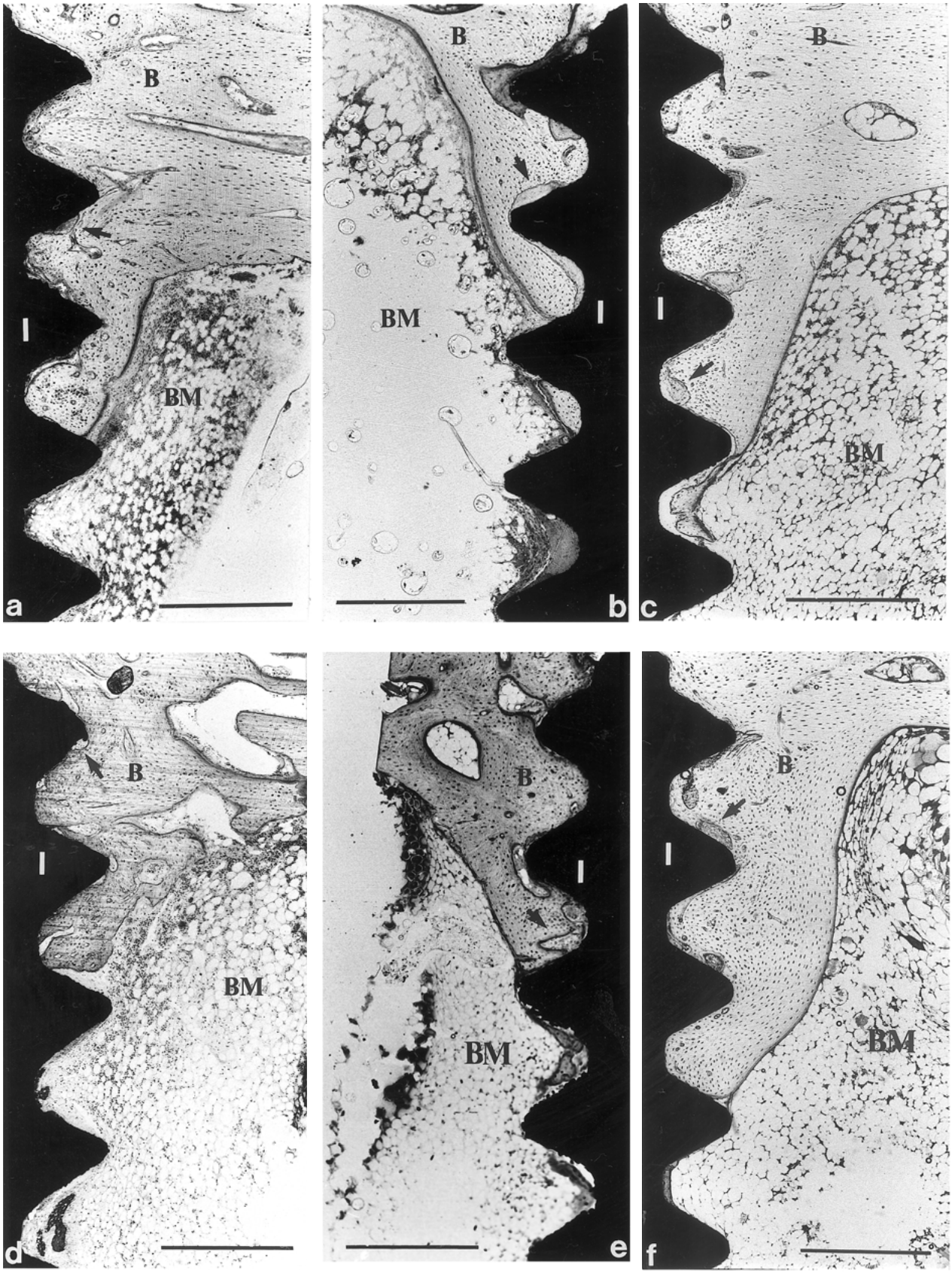

Figure 6 Light micrographs of ground sections prepared from bone–implant specimens. Hf specimens after (a) 6, (b) 12, (c) 24 weeks and Ti specimens after (d) 6, (e) 12 and (f) 24 weeks. Note bone (B) close to the implants (I). Bone marrow (BM). Some areas with soft tissue (arrows) are located adjacent to the implant suface. Bar = 500 μm.

tionships between implant material properties and the specific events which constitute these biologic processes are not known in detail. It is therefore yet difficult to deduce with high precision the biocompatibility of a material from animal experiments and to predict the long-term performance of a material in a clinical application. Among the quantifiable parameters which are commonly used to evaluate the subsequent cellular and tissue response after implantation of a material are: the plasma protein exudation and accumulation, inflam-

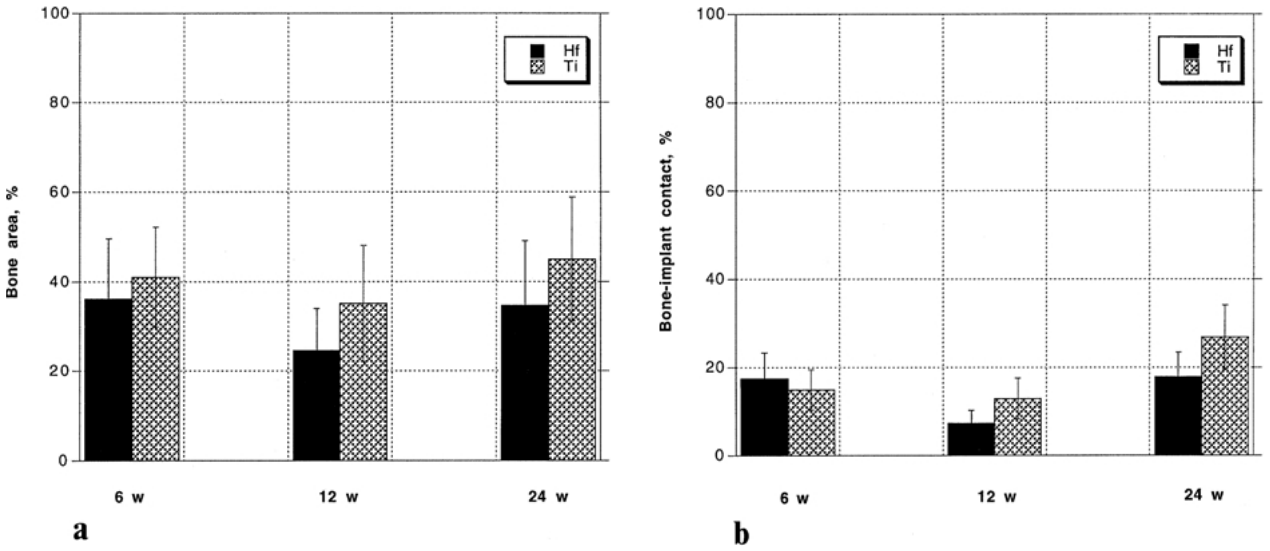

Figure 7 Bone morphometry of ground sections for all observation periods. (a) Percentage of total mean ± SEM bone area within the threads of Hf and Ti implants. (b) Percentage of total mean bone contact within the threads of Hf and Ti implants.

matory cell migration and distribution, and the development of the surrounding fibrous capsule (fibrous capsule width) in soft tissues, alternatively a regeneration of bone (bone area, volume and contact in association with implant) in osseous sites. In the present study, the results show that solid Ti inserted in soft tissues was surrounded by mainly macrophages, predominating close to the implant surface, and fibroblasts. An initial fluid space became reduced with time, indicative of a transient edema. Further, a fibrous capsule developed around the material. The observations agree with previous studies on titanium (reviewed in [12, 13]). A major morphological observation was that the tissue responses were similar around Ti and Hf with no statistically significant differences observed for any of the parameters in soft and hard tissues and in two different animal species.

Using the same experimental model in rats, other materials, including PTFE and polyacetal had a different plasma protein distribution, relatively greater inflammatory cell accumulation, higher LTB4 content and extensive fibrous overgrowth of the rod portion of the implant and a thicker fibrous capsule [7, 14–16]. Based on a series of studies in an experimental rat model using another implantation site (s.c. dorsum), it was found that different chemically functionalized gold surfaces, having similar surface roughness, induced different plasma protein adsorption patterns, inflammatory cell recruitment, distribution and respiratory bursts, but did not influence the cytokine concentrations and the fibrous capsule thickness [17]. Thus, the chemical properties of the material surface appear to modulate specific events during at least the early time stage of inflammation around materials implanted in soft tissues. On the other hand, a porous or textured surface has been found to modify the cellular behavior, including the number of macrophages and the fibrous capsule thickness [18, 19]. The present study used Ti and Hf with similar surface topography and it cannot therefore be excluded that a differently textured Hf or other forms of the material would elicit a different response, since both material surface and biological factors promote the differentiation of macrophages to multinuclear cells [20].

Comparative long-term (up to 1 year) studies on metals in rabbit muscle have shown the development of a fibrous capsule around all materials, with Zr having a (slightly) thicker capsule than Ti [21, 22]. Hf has not yet been examined in such long-term experiments.

In bone, a trend toward a lower degree of bone-implant contact was observed for Hf implants but no statistical differences were detected. Previous studies which have compared the response in cortical bone to Ti and Zr have revealed only minor differences in the response to these two metals. Ultrastructural observations have indicated that the layer of non-collagenous amorphous material located between the implant surface and the calcified bone was appreciably thicker around Zr than around Ti [4, 5]. Recently a somewhat better interfacial response to Zr than Ti implants was reported [6]. Histomorphometric data [5] have shown that Ti and Zr had a similar bone area and bone-implant contact after 1 and 6 months, in contrast to gold (Au) which had a lower degree of bone apposition and poor mineralization at the interface. Thus, at least Ti and Zr have qualitatively similar surface properties and share positive effects on the bone tissue response after implantation.

Previous studies on Hf have shown that a single intradermal injection in the external ears of mice of Hf oxychloride as well as Zr oxychloride induced benign dysplasia of the cartilage, whereas no cartilaginous changes were observed in control animals given similar injections of experimental metal carcinogens, such as salts of Aluminum, beryllium, cadmium, chromium, cobalt and nickel [23]. A preferential localization of Hf in macrophages in lymph nodes was demonstrated after intraperitoneal injection of soluble hafnyl chloride in rats [24]. Similar observations were made with Zr salt [25], TiO_2 particles injected intravenously [26] and Ti particles after placement of plasma-sprayed screw implants in the maxillofacial region [27]. However, no evident pathological changes were observed in any of these published *in vivo* investigations using particulates. Together, the present and these published experimental observations indicate similar biological responses to Group IV metals.

The present observations suggest that Hf, having a surface oxide, is an interesting material for studies on

protein adsorption and cell adhesion and activation, including comparative studies with neighboring metals Ti, Zr and Au (lacking surface oxide). Together, the available data suggest that Hf is a highly interesting metal for biomedical applications.

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