H_2O_2 production by cells on titanium and polystyrene surfaces using an *in vivo* model of exudate and surface related cell function

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The determination of secreted levels of reactive oxygen species by implant-adherent cells *in vivo* is required for understanding of the role(s) of such reactive oxygen species for the tissue response around medical devices. A model with subcutaneous implants of c.p. titanium (Ti) or polystyrene (PS) (cell culture grade) inserted on the back of rats were used. Implants and associated cells were retrieved and assayed after 1, 3, 5, 7, 14, 21 and 28 days. Morphological analysis of exudate cells showed that polymorphonuclear leukocytes (PMN) predominated after one day whereas macrophages were predominant after three days. The number of implant-adherent cells, as reflected by measurement of DNA, decreased with time. Ultrastructural observations showed that macrophages were predominant cells in contact with the implant surface. Measurement of hydrogen peroxide (H_2O_2) secretion by implant-adherent cells during 40 min incubation *ex vivo* revealed a constitutive generation of 40–400 pmol $H_2O_2/\mu g$ DNA, depending on implantation time. Stimulation with protein kinase C agonist phorbol myristate acetate (PMA) caused an increased H_2O_2 generation by adherent cells at early (up to five days) but not later (7–28 days) time periods. No major differences between Ti and PS were observed. Taken together, these findings show that Ti and PS implant-adherent cells secrete H_2O_2 under *in vivo* conditions. Further, a reduced capacity to mount an enhanced H_2O_2 secretion upon stimulation was demonstrated at late time periods. The role of this mediator for biocompatibility remains to be established.

1. Introduction

The interface between the surface of an implant and the surrounding tissue is the scene of encounter between the introduced non-biological component and the defence system of the body. The outcome of this interaction has a major influence on the possibility to achieve and maintain tissue integration [1]. Factors influencing this interaction include material composition, implant design, surface properties, implant location, state of the host bed, surgical technique and mechanical loading [1–5].

Reactive oxygen species (H_2O_2, O_2^- and OH^-) are products of cell metabolism and are produced by the NADPH oxidase in most cell types. Activation of phagocytic cells by appropriate stimuli leads to the assembly of the active NADPH complex at the plasma membrane [6], The production of reactive oxygen species by phagocytes is crucial for their microbicidal activities [7, 8], whereas in low concentrations reactive oxygen species are implicated in ''non-toxic'' signaling [9, 10]. Toxic oxygen metabolites have been implicated in the pathogenesis of numerous inflammatory diseases [8]. High concentrations of H_2O_2 cause cell damage as evidenced by DNA damage, inactivation of aldehyde and lactate dehydrogenases, decrease in lactate content [11] and stimulate vascular endothelial growth factor, crucial for wound healing [12]. Extracellularly secreted H_2O_2 induce apoptosis in monocytes *in vitro* [13].

The macrophage is the major cell type in contact with the surface of implants located in soft tissues [14–17]. Differentiation of monocytes to macrophages *in vitro* is associated with a decrease in the production of both reactive oxygen species and scavengers [18–20]. Interleukin-4 (IL-4) promotes differentiation and inhibits agonist-stimulated O_2^- production in human monocytes [21] and inhibits interferon-gamma (IFN-γ)-mediated production of H_2O_2 in cultured human monocytes [22].

The secreted levels of H_2O_2 and other reactive oxygen species by cells in the implant–tissue interface may therefore be of importance for the tissue response at implanted materials. However, to the authors' knowledge no previous studies have been focussed on the analysis of H_2O_2 at implant surfaces *in vivo*. Previous studies *in vitro* have shown that the production of reactive oxygen species by phagocytic cells is dependent

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on the cells being adherent or suspended, the type of substrate and the type of stimulus [23–26]. A dose-dependent increase in H_2O_2 secretion was observed in short-term culture of polystyrene (PS) particle-stimulated human monocytes but this response was abrogated after a prolonged culture period concomitant with maturational phenotypic alterations [27].

A major reason for the scarcity of *in vivo* data on the fine structure and activities of cells, including secreted reactive oxygen species, at the interface between material and tissue is related to the inaccessibility of the interface zone. During the initial weeks after implant insertion in soft tissues, a fluid space which contains cells and proteins, is present between the implant surface and reorganizing tissue [28–31]. The fluid space can be sampled and its contents analyzed in models using hollow implants [32, 33]. On the other hand, the activities of implant-adherent cells have yet remained unresolved.

The aim of the present study was to determine the amount of *in vivo*/*ex vivo* produced constitutive and agonist-stimulated H_2O_2 by cells adherent on two different material implant surfaces. The number of non-adherent cells found in the fluid space was also of interest, reflecting cell recruitment to different materials. Solid implants were inserted subcutaneously in rats and retrieved after different time periods. The oxidative metabolism of implant-adherent cells was determined. Cell numbers was evaluated by measurement of the DNA content on the solid implants. Ultrastructural observations of implant-adherent cells was performed on ultrathin sections prepared after application of an electrolytical dissolution technique [34].

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (6 rats/group), weighing 250–300 g and fed on standard pellet diet and water *ad libitum* were used. The rats were anesthetized with intraperitoneal injections (0.3 ml) of a solution composed of sodium pentobarbital (Mebumal Vet®, Nordvacc, Sweden) (60 mg/ml), 0.9% saline, and diazepam (Apozepam®, Apothekarnes Laboratorium AS, Norway) (5 mg/ml) in 1 : 1 : 2 volume proportions. The experiments were approved by the Ethical Committee for Laboratory Animals in Göteborg, Sweden.

2.2. Implants

Circular PS implants (diameter 16 mm, thickness 1.2 mm) were prepared by sawing from the bottoms of polystyrene tissue culture wells (Nunc®, Denmark). Ti implants (cp. titanium, grade 1, Edstraco AB, Sweden) of the same dimensions were made by machining in a lathe. The PS implants, with the tissue culture treated side (according to the supplier) marked, were wiped with a clean cotton towel and cleaned by ultrasound in absolute ethanol (3 × 10 min). The PS implants were implanted with the tissue culture treated surface towards the skin. The Ti implants were cleaned by trichloroethylene, rinsed in absolute ethanol and thereafter cleaned in ultrasound (15 min), in absolute ethanol (3 × 10 min). The implants were finally rinsed and kept in sterile saline until surgery.

2.3. Implantation procedure

After shaving, the dorsal skin was cleaned with 2% Jodopax®. About 20 mm long skin incisions were made bilateral about 15 mm from the midline. The implants were placed in small subcutaneous pockets prepared by careful dissection avoiding bleeding. The pockets, three on each side were separated from each other by about 15 mm wide zone of intact tissue. Each pocket was closed with three skin sutures (Vicryl® Ethicon, Sweden). Altogether six implants, three on each side of the midline, were inserted in each rat. The locations for the two types of implants were systematically alternated. No implant pockets were in contact with each other.

2.4. Retrieval procedure

After 1, 3, 5, 7, 14, 21 and 28 days the implants were retrieved. At each time period (6 rats/time interval) rats were anesthetized, shaved and the skin was carefully cleaned with Jodopax®. The rats were killed by an i.p. overdose of pentobarbital (60 mg/ml). A midline skin incision was made in the dorsal midline and the skin with the subcutaneous tissue containing the implant was folded laterally. The fibrous capsule surrounding the implants was cut with a pair of fine scissors, and the implant retrieved.

The content of the cavity present after removal of the implant was collected by repeated aspiration (×5) with 0.5 ml Hank's balanced salt solution (HBSS) with Ca^{2+}. The aspirate was kept on ice. Cells were counted in a Bürker chamber after staining with Türk's stain and the cell viability (always > 95%) was determined by the exclusion of Trypan Blue. In order to reduce the possible effect of pentobarbital on cell activity, the excision procedure was performed as rapidly as possible. Cytocentrifuged slides stained with Giemsa were prepared for differential counting. The implants were put in sterile polystyrene tissue culture dishes (Nunc®, Denmark) with 2 ml sterile HBSS (with Ca^{2+} and Mg^{2+}), pH 7.4 and kept on ice.

2.5. Measurements of H_2O_2

The production of H_2O_2 by cells associated with the implant surfaces on both sides was measured with the homovanillic acid (HVA) fluorescence assay [35]. The implants were placed in 2 ml HBSS (with Ca^{2+} and Mg^{2+}), pH 7.4, supplemented with 4.4×10^{-4} M homovanillic acid (HVA) and 0.5 U/ml horseradish peroxidase (HRP, Sigma, USA). The dishes were incubated at 37 °C for 40 min under constant shaking. After incubation, the medium was chilled and measured in a Perkin-Elmer LS luminescence spectrometer (Norwalk, USA). The excitation wavelength was set at 315 nm, and the emission wavelength at 450 nm. The amount of H_2O_2 present was determined from standard curves produced from measurements of buffer samples with known amounts of H_2O_2. The stimulatory effect by phorbol 12-myristate 13-acetate (PMA, Sigma, USA) on

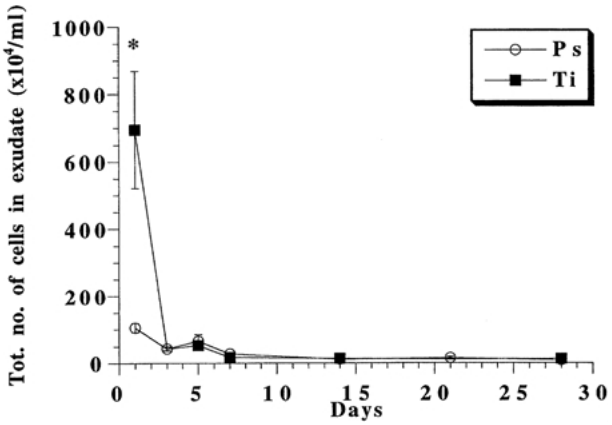

Figure 1 The number of leukocytes ($\times 10^4$/ml) in the exudate around titanium (Ti) and polystyrene (PS) implants 1–28 days after implantation. Data is given as mean $\pm$ SEM. The mean is based on the values obtained from six rats at each time period. In each rat the mean values were calculated from the exudates around three implants of the same type ($*P < 0.02$).

H_2O_2 production was examined by adding PMA for 40 min. (10^{-6} M final concentration) to parallel dishes followed by measurement of H_2O_2 as described above.

2.6. Measurements of DNA

The DNA content of the cells adhering to both sides of the implants was determined by a fluorescence assay [36]. In brief, after aspiration of the medium for H_2O_2 measurements, 2 ml 5×10^{-2} M sodium phosphate buffer with 2×10^{-3} M EDTA and 2 M NaCL was added to the dishes containing the implants. Thereafter, the recovered implants were frozen at $-20\,^{\circ}$C. After thawing and ultrasound treatment (20 s on each side of the implant), 200 µl of the buffer solution containing cells was aspirated and added to 2 ml of 5×10^{-2} M sodium phosphate buffer with 2 M NaCl, supplemented with 1 µg/ml Hoechst 33258 (Sigma, USA). After incubation in room temperature for 15–30 min the buffer medium was aspirated and measured in the luminescence spectrometer. The excitation wavelength was set at 360 nm, and the emission wavelength at 450 nm. Standard curves were produced from measurements of buffer samples with known amounts of DNA (5–500 µg/ml) (Sigma, USA).

2.7. Morphology

In each rat one implant of each material was fixed by immersion in 2.5% glutaraldehyde in 5×10^{-2} M sodium cacodylate buffer, pH 7.4, overnight and then postfixed in 1% OsO_4 for 1 h. After dehydration in a graded series of ethanol, the implants were embedded in epoxy resin (Agar 100, Agar Aids, UK). Ultrathin sections of cells on the PS implants for transmission electron microscopy were either obtained by cutting through the PS or after fracturing the PS away from the epoxy resin. The Ti implants were treated with an electrochemical procedure [34]. By this method the bulk metal is removed while the surface oxide layer, in contact with the tissue and thin enough to be cut by an ultramicrotome remains. In brief, the resin-embedded

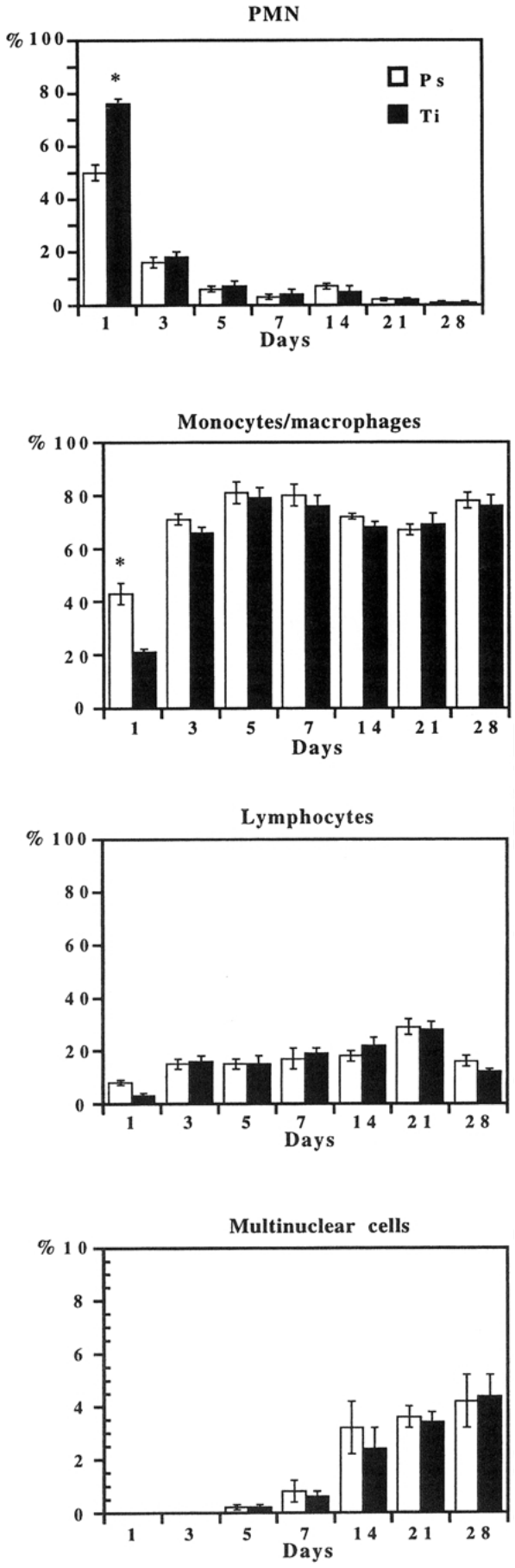

Figure 2 The percentage of different cells in the exudates around titanium (Ti) and polystyrene (PS) implants 1–28 days after implantation. Data is given as mean $\pm$ SEM. The mean is based on the values obtained from six rats at each time period. In each rat the mean values were calculated from the exudates around three implants of the same type. On each cytocentrifuge preparation 10^2 cells were counted ($*P < 0.02$).

implants were divided in four parts by sawing. One part of the divided implant was exposed by grinding and another part connected to an electrode. The surface of the specimen except for the ground and exposed surface of the implant, was insulated by lacquer (Lacomit, Canning & Co., UK) and served as anode when mounted in the electropolishing equipment. The specimen was surrounded by an electrolytic platinum cathode and electropolishing performed in an electrolyte (5% perchloric acid, 35% *n*-buthanol and 60% methanol) under cooling ($-30\,^{\circ}C$) and vigorous stirring. When the bulk part of the metal was removed, the specimen was disconnected from the cell, rinsed in water and re-embedded in epoxy resin.

Semithin sections for light microscopy (LM) were cut on an ultramicrotome using glass knives. Selected areas were cut using a diamond knife and stained with uranyl acetate and lead citrate for transmission electron microscopy (TEM) (Phillips EM 400).

2.8. Statistics

Wilcoxon range sum test was used as statistical test, $^{*}P < 0.02$.

3. Results

3.1. Analysis of cells in the exudate

Analysis of the cellular content in the exudates around PS and Ti implants 1–28 days after implantation showed that the highest number of cells was detected during the early phase of healing (before five days) (Fig. 1). The largest amount of cells was found in exudates around Ti implants one day after insertion. After seven days and at later time periods few cells were retrieved from the exudates.

The majority of cells in the exudate was leukocytes. In cytocentrifuged preparations, PMN predominated after one day (Fig. 2). At this time point PMN constituted a markedly larger proportion of the leukocytes around Ti (76%) than PS (49%) implants, respectively. After three days the majority of cells in the exudates around both Ti and PS was monocytes/macrophages. The major proportion of these cells was small monocytes. The proportion of lymphocytes in the exudate increased during the observation period (the highest percentage, 28%, was observed after 21 days) (Fig. 2). Few multinuclear cells were detected during the first week. During the period, from 14 days to 28 days, the multinuclear cells constituted about 4% of the total number of exudate cells (Fig. 2).

3.2. Analysis of implant-adherent cells

The determination of DNA, reflecting the number of adherent cells, showed different time-dependent patterns for the two type of implants (Fig. 3(a)). These patterns were not influenced by the addition of PMA (Fig. 3(b)). For Ti implants a very high amount of DNA was found at day 1 with a gradual decrease to very low levels at 28 days. PS implants had peak levels at day five; the values then gradually decreased to very low levels at 28 days.

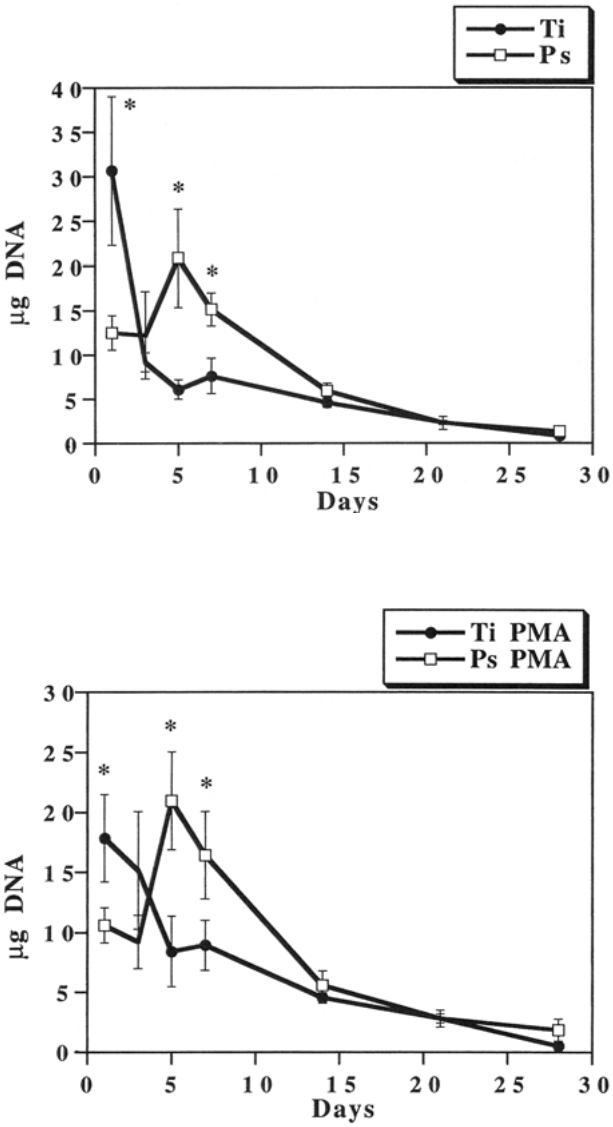

Figure 3 (a) DNA content (μg) in cells on titanium (Ti) or polystyrene (PS) implants. Data is given as mean ± SEM. Values are the mean of six experiments ($^{*}P < 0.02$). (b) The effect of PMA (10^{-6}M) on DNA content in cells on titanium (Ti) or polystyrene (PS). Data is given as mean ± SEM. Values are the mean of six experiments ($^{*}P < 0.02$).

3.3. Electron microscopy

PS implants retrieved after seven days were covered by 1–3 layers of cells (Fig. 4). No differences were seen between the two implant surfaces. The innermost layer in contact consisted either of multinuclear giant cells or large epitheloid macrophages. The latter had large, cuboidal cell profiles and contained large amounts of rough endoplasmatic reticulum in the cytoplasm. Phagocytic vacuoles were usually not present.

When several layers of cells were present (Fig. 4(a)), these were kept together by deposits of fibrin. Occasional fibroblasts were present but these cells were invariably separated from the surfaces by at least one layer of macrophages or multinuclear giant cells.

On Ti implants removed after seven days, 1–3 layers of macrophages were attached to the surface (Fig. 5(a),(b)). Multinuclear giant cells and epitheloid macrophages were rare and the cells attached had a smaller cell body and with few phagocytic vacuoles in their

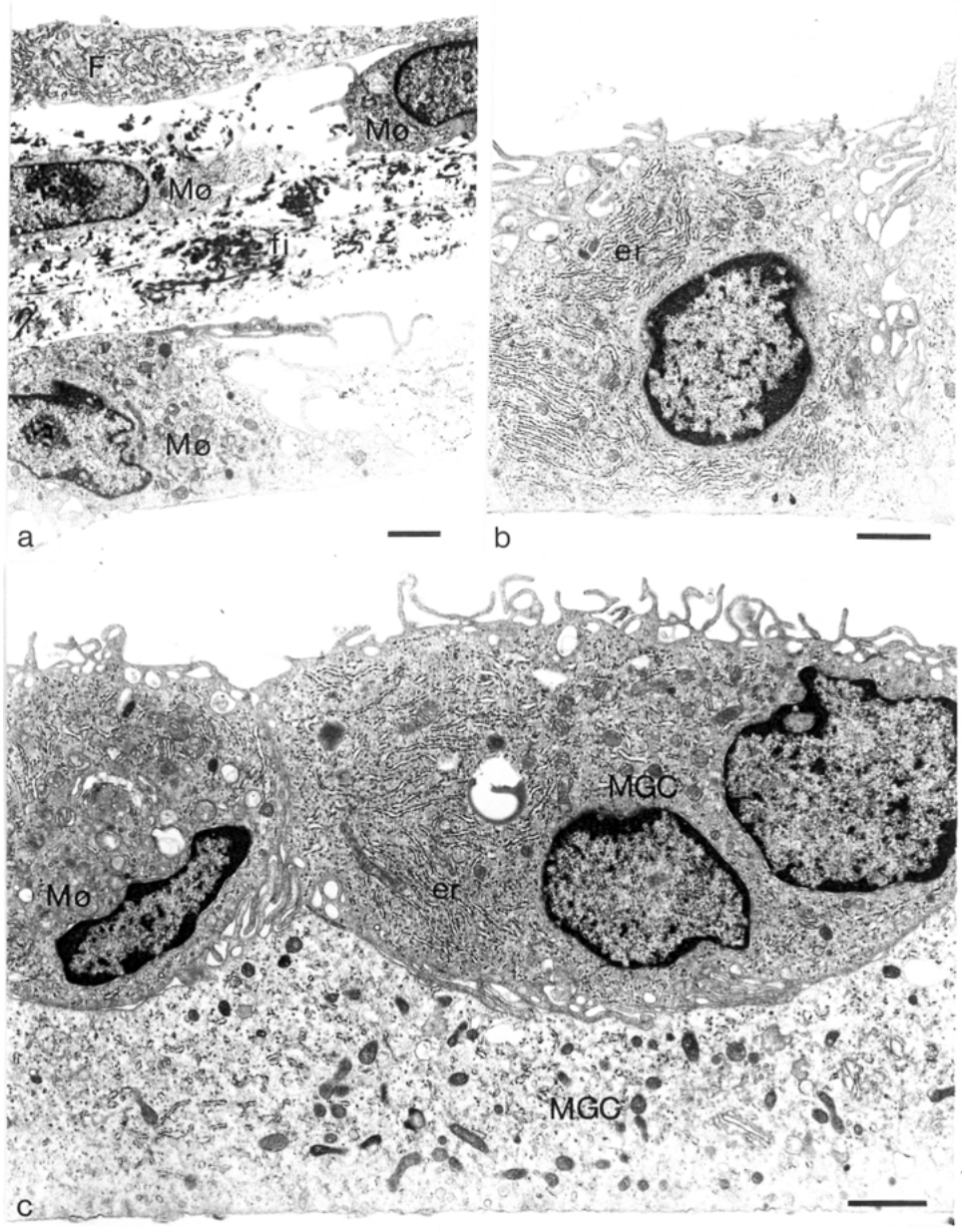

Figure 4 Mount of electron micrographs of cells adhering to polystyrene (PS) implants removed after seven days. The implant is located in the basal part of the micrographs. (a) Often several layers of cells, which were kept together by fibrin strands (fi), were observed. Macrophages (Mø) was by far the most common cell type but occasional fibroblasts (F) were detected. Fibroblasts were always separated from the implant surface by several layers of macrophages. × 4.500 (bar = 2 μm). (b) A large epitheloid macrophage attached to the polystyrene surface. The cell contains large amount of rough endoplasmatic reticulum (er). × 6.500 (bar = 2 μm). (c) Multinuclear giant cells (MGC) and a macrophage (Mø) adhering to polystyrene. × 7.000 (bar = 2 μm).

cytoplasm. After 21 and 28 days (Fig. 5(c)–(f)) appreciably fewer cells remained on the implants after their removal. On Ti implants (Fig. 5(c),(d)) macrophages, and only occasional multinuclear giant cells, were present. On PS implants flat, multinuclear cells (Fig. 5(f)) or macrophages (Fig. 5(e)) were found.

3.4. Production of hydrogen peroxide

Irrespective of observation period, a low spontaneous H_2O_2 production was found in cells adherent to Ti surfaces (Fig. 6(a)). The spontaneous H_2O_2 production was only slightly higher in cells on the PS surfaces retrieved during the initial five days, increasing to day 15 (Fig. 6(a)). PMA (10^{-6} M) was found to increase the generation of H_2O_2, in cells on Ti and PS surfaces during the first five days (Fig. 6(b)). The absolute amounts of constitutively generated H_2O_2 by material surface-associated cells, obtained after 1–28 days were 500–1000 pmoles and 500–2500 pmoles during 40 min incubation for Ti and PS, respectively. After PMA-stimulation levels were 500–3000 pmoles and 500–3500 pmoles for Ti and PS, respectively.

When correlating the amount of H_2O_2 to the DNA content (Fig. 7), a slight increase in the H_2O_2 production at day five on the titanium surface was shown and the cells were capable of increasing the production of H_2O_2 when stimulated by PMA.

Analysis of the kinetics of H_2O_2 generation per cell (DNA) revealed a similar progressive increase up to day 14 and day 21 on PS and Ti, respectively. At day 28 low amounts of H_2O_2 generation were detected. In general, the constitutive levels of generated H_2O_2 were ~ 40 pmol H_2O_2/μgDNA $\rightarrow 400$ pmol H_2O_2/μgDNA,

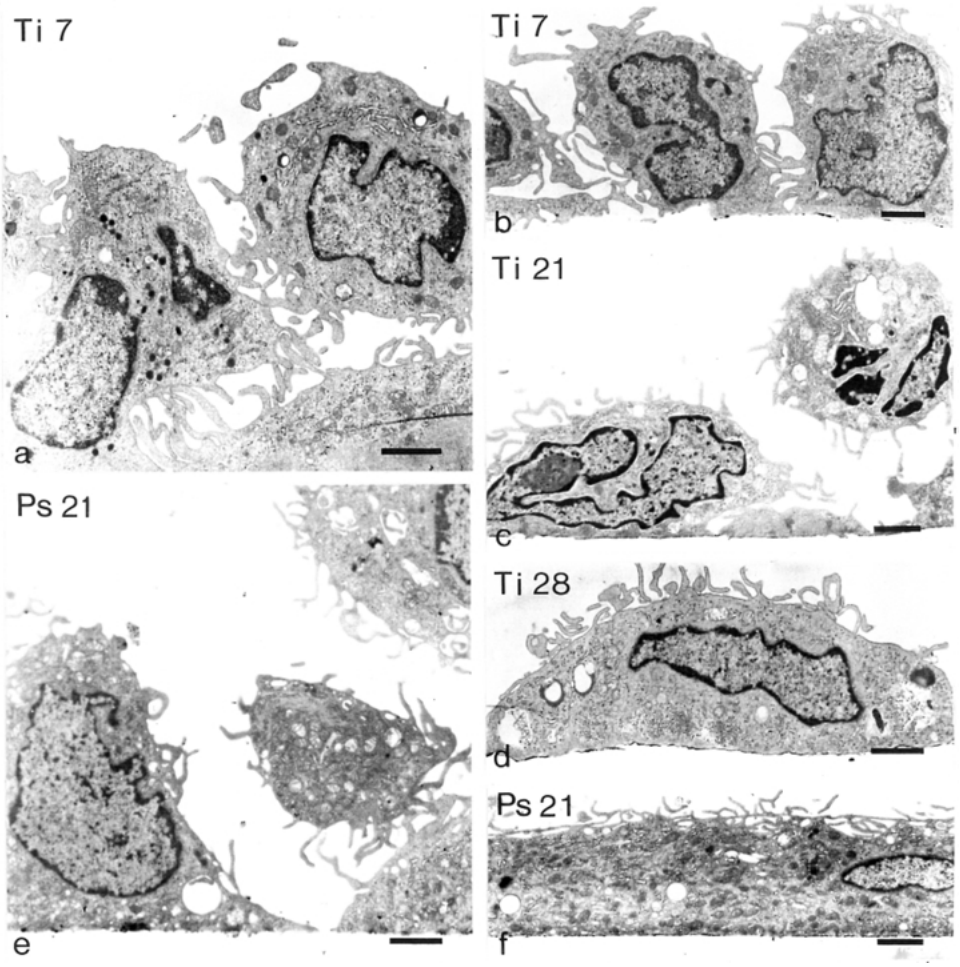

Figure 5 Mount of electron micrographs of cells at polystyrene (PS) and titanium (Ti) surfaces retrieved at the times (days) indicated. (a) Macrophages located close to the surface of titanium seven days after implantation. Two of the cells are adherent to the surface. Note accumulation of actin filaments (af) in one of the macrophages attached to the substratum. × 5.000 (bar = 2 μm). (b) Macrophages with numerous long, cytoplasmic extensions are located on the implant surface. × 3.600 (bar = 2 μm). (c, d) Macrophages adjacent to titanium implant have an inactive appearance. (c) × 4.000; (d) × 4.500 (bar = 2 μm). (e) Macrophages on polystyrene display a variable morphology. Their size and number is considerably reduced as compared to that observed after seven days. × 4.500 (bar = 2 μm). (f) Portion of flat multinuclear giant cells on polystyrene (after 21 days). × 4.000 (bar = 2 μm).

depending on implantation time. The PMA-stimulated generation of H_2O_2 resulted in ∼ 100–700 pmol/μgDNA, depending on implantation time.

4. Discussion

In the present study, using a rat s.c. model, an initial high recruitment of leukocytes and adhesion of cells to the surface was found with both PS and Ti implants. However, after an early sharp increase in cell numbers observed with Ti and a delayed pattern found with PS, similar cell numbers were observed after three days (exudate) and 14 days (surface). The reason for the early differences may be related to different material surface properties and initial differences in protein adsorption with subsequent effects on cell modification. This assumption is supported by *in vivo* observations that implant surface chemical properties influenced plasma adsorption, cell recruitment and distribution but not viability and release of proinflammatory cytokines [37, 38]. The majority of the cells in the exudate were leukocytes, of which PMN were predominant after one day. However, after three days the majority of cells were monocytes and macrophages. The total cell number in the exudates decreased rapidly, and after day seven very low cell numbers were found also in agreement with previous studies [39]. Our ultrastructural observations after seven days showed that macrophages were the most common cells on the surface of the retrieved implant. These observations corroborate previous morphological and morphometrical studies using solid titanium in the abdominal wall of the rat [28]. In a previously described chamber model [32], where the interior of the chamber is communicating with the surrounding fluid phase via pores, the exudate is of another character than around solid implants. The PMN is the dominant cell type both inside the hollow chamber and in the surrounding tissue. Thus, the present model and means of exudate retrieval seems to be more adequate for analysis of the fluid space content around implants.

The spontaneous production of H_2O_2 in Ti and PS adherent cells during a 40 min incubation period was in the 40–400 pmol/μg DNA range, depending on implantation time. These values were higher than those detected with human monocytes adherent on PS *in vitro* as measured after 24 h (about 2 pmol/μg DNA) [27] and with cells adherent on Ti and PTFE in a murine model after one and six days [40]. Possible explanations for this difference are species differences and differences in culture conditions (*in vitro*/*ex vivo*). Since addition of

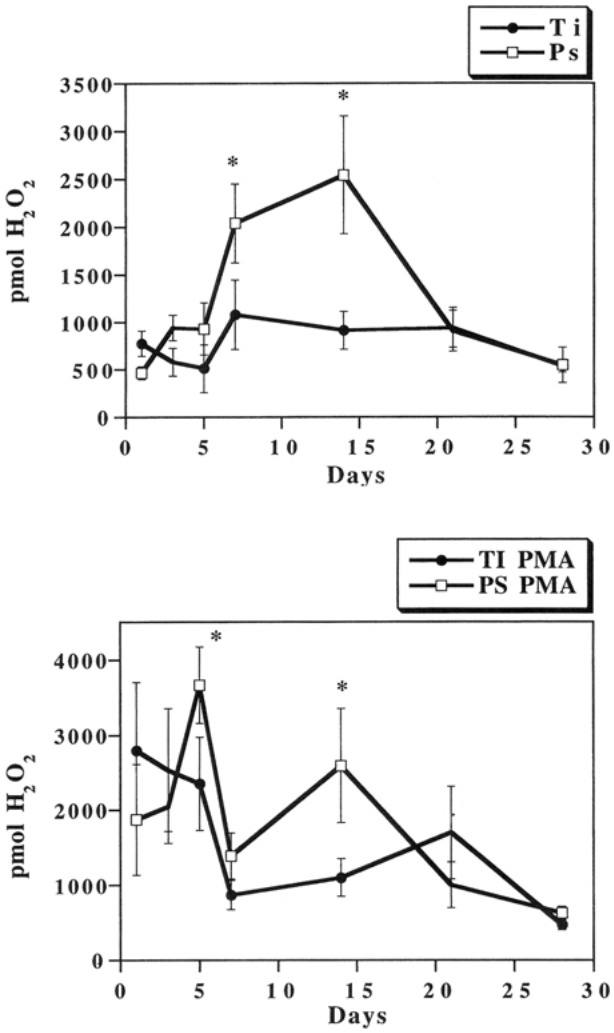

Figure 6 (a) The spontaneous generation of H_2O_2 (pmol H_2O_2) in cells grown on titanium (Ti) or polystyrene (PS). H_2O_2 was measured in the assay buffer at the end of a 40 min incubation period. Data is given as mean ± SEM. Values are the mean of six experiments ($*P < 0.02$). (b) The effect of PMA (10^{-6}M) on H_2O_2 generation pmol (H_2O_2) in cells grown on titanium (Ti) or polystyrene (PS). H_2O_2 was measured in the assay buffer at the end of a 40 min incubation period. PMA was added at the beginning of the period. Data is given as mean ± SEM. Values are the mean of six experiments ($*P < 0.02$).

PMA in the present study and PS particles to adherent cells in the *in vitro* model [27] markedly increased the H_2O_2 production it may be concluded that the leukocytes at these implant surfaces were not maximally metabolically activated. It was apparent that phenotypic changes of material surface adherent macrophages both *in vitro* and *in vivo* was correlated to changes in the ability to produce H_2O_2. This was evident as early as after 48 h culture on PS *in vitro* [27], resulting in lack of responsiveness to particles.

The present *in vivo* observations are in agreement with the observations made on the release of superoxide anion (O_2^-) by macrophages on subcutaneously implanted glass in mice [41]. In the latter study, macrophages on coverslips implanted for 14 days or longer secreted a protease-sensitive, heat-stable factor with deactivating/downregulatory effect on O_2^- release by PMA-stimulated cells. A crucial question is if the secretion of reactive oxygen species is modified by different implant material properties. On one hand, a higher absolute level of H_2O_2 was produced by cells obtained from PS surface during the transient inflammatory phase (7–15 days). However, this level was an effect of a concomitant higher, transient number of implant-adherent cells on the PS surface in comparison with Ti. On the other hand, neither the amount of H_2O_2 generated per cell differed between cells on the two model surfaces, nor did the inhibition of responsiveness to PMA-stimulation differ between cells on the two surfaces. Previous studies on the early phase of inflammation (3 h and one day) showed that cells on functionalized (–OH), hydrophilic implant surfaces respond with a higher generation of extracellularly secreted reactive oxygen species than functionalized ($-CH_3$), hydrophobic surfaces [38]. Previous studies *in vivo* [40] and *in vitro* [23–26] have also shown that the production of reactive oxygen species by phagocytes and their ability to respond to exogenous stimuli is dependent on the cells being adherent or suspended, the type of substrate and the type of stimulus. However, although a plausible hypothesis, there is yet no *in vivo* evidence to suggest that cells on biocompatible materials differ from those on less tolerable/cytotoxic materials with respect to the generation of reactive oxygen species and/or their ability to mount an increased generation of such species upon stimulation.

The design of the *in vivo* experiments, focussing on H_2O_2 secretion and morphology of implant-adherent macrophages, did not permit simultaneous assaying of cytokines. On the background of a demonstrated decrease of proinflammatory cytokines (IL-1α, IL-1β and TNF-α) during the initial month of healing around implanted biomaterials [42] it could be of great interest to determine the production of other cytokines and their effects on the release of reactive oxygen species. This is suggested by the observation that the proportion of lymphocytes and multinuclear cells slowly increased in the present *in vivo* study. *In vitro*, lymphocyte-derived cytokines have been shown to stimulate monocyte adhesion, macrophage maturation and fusion of macrophages to multinuclear giant cells [43–47]. IL-4 and IFN-γ, secreted by T helper lymphocytes subsets have multiple activating and inhibiting effects, respectively, on monocyte/macrophages [48–51], including IL-4 inhibition of the secretion of reactive oxygen species [22].

The present findings of a spontaneous generation of reactive oxygen species during the inflammatory and healing phase as well as a time-dependent reduction in the radical production capacity may have implications for the tissue response as well as for the efficiency whereby microorganisms are eliminated. The production of reactive oxygen species by phagocytes is crucial for their microbicidal activities [7, 52], and in numerous inflammatory diseases, toxic oxygen metabolites have been implicated in the pathogenic mechanisms [8]. Studies *in vitro* have also shown that cellular activity may be modulated by H_2O_2 and O_2^-, depending on the concentrations, thus expanding the properties of reactive oxygen to those of other cellular messengers (reviewed in Gamaley and Klyubin [6] and Rhee [53]). Low concentrations (1 μM) of H_2O_2 stimulated the proliferation of fibroblasts, whereas higher concentrations (100–400 μM) induced senescence or apoptosis [9, 10].

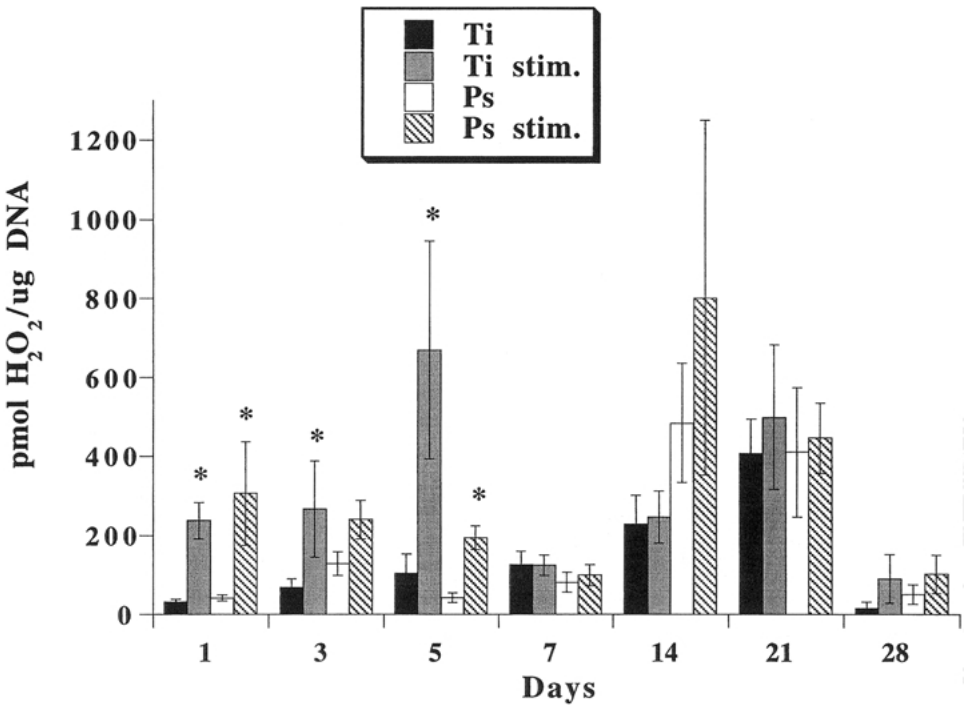

Figure 7 The generation of H_2O_2 per DNA content (pmol/μg) in unstimulated or PMA stimulated cells on titanium (Ti) or polystyrene (PS) (*$P < 0.02$)

Hitherto, pharmacological intervention aiming towards a reduction of inflammation and the fibrous capsule formation has not been successful. It is therefore interesting that low molecular weight (500–600 Da) superoxide dismutase (SOD) mimics (SODm), which inhibit inflammation [54], covalently bound to UHMWPE, polyether-urethane urea and tantalum, reduced the material surface oxidation, the number of macrophages and multinuclear cells on the surface and the thickness of the surrounding fibrous capsule [55]. Taken together, *in vivo* observations now indicate an important role of reactive oxygen species for the tissue response at biomaterials.

In vitro studies have shown an oxidation of Ti with H_2O_2 concentrations down to < 1 mM, resulting in the formation of Ti peroxy compounds possessing anti-inflammatory and bactericidal properties [56]. The present *in vivo* results do not show that Ti surface adherent macrophages produce H_2O_2 in a similar amount, even after the activation of the cells with the non-receptor dependent PMA. On the other hand, it cannot be excluded that both the intimate relation between macrophages and Ti, and the formation of a dense, fibrous capsule [29, 57] could promote a local, high concentration of reactive oxygen species with effects on the TiO_2 [56]. The generation of reactive oxygen species by macrophages during the process of ''normal healing'' is likely to be different from that observed during the process of implant loosening when a large amount of wear debris accumulate at the implant site. However, direct measurements of reactive species in such *in vivo* situation have not been reported.

In summary, these findings show that implant adherent macrophages produce H_2O_2. Further, our *ex vivo* data indicate that the oxidative metabolism may be down-regulated. At present, it is not known if this modulation of cell function is restricted to certain materials with specific surface properties and how these phenomena are related to the biocompatibility of a material.

Acknowledgments

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