In vivo/*ex vivo* cellular interactions with titanium and copper

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Machined, commercially pure titanium (Ti) disks were coated with approximately 400 nm copper (Cu) by physical vapor deposition or left uncoated. The kinetics of inflammatory cell recruitment, distribution and viability was evaluated around Ti, Cu, and in sham sites after 1, 3, 12, 18, 24, and 48 h in a rat subcutaneous (s.c.) model. Further analysis of the cells on implant surfaces was performed by *ex vivo* incubation of the disks. Ti and Cu stimulated an increased recruitment of inflammatory cells in comparison with sham sites. A markedly higher amount of cells, predominantly polymorpho-nuclear granulocytes (PMN), was detected around Cu after 18 h and onwards. More cells were found at the implant surfaces than in the surrounding exudates after 18 h. The total amount of lactate dehydrogenase (LDH), an indicator of plasma membrane injury, was higher in Cu exudates after 18 h in comparison with Ti and sham. In contrast, no differences in the proportion of dead cells (trypan blue dye uptake) were detected in the exudates. Further, LDH levels were higher around Ti than Cu during the initial 18 h of *ex vivo* incubation. The results of this study indicate that the early inflammatory process associated with a cytotoxic material in soft tissues is largely attributed to the induction of a markedly strong and prolonged chemotactic response. In contrast, this process is characterized by a higher amount of inflammatory cells around a biocompatible material than in sham sites, but with a transient course and total LDH similar to sham sites.

1. Introduction

The migration and accumulation of leukocytes constitutes a hallmark of the inflammatory response at an implant site. The surgical trauma and the presence of the implant stimulate leukocytes to migrate to the implant-tissue interface. The chemotactic response around titanium (Ti) is transient and lower in magnitude than for several other implant materials, which is of importance when designating a material the properties of biocompatibility (reviewed in Holgers *et al*. [1]).

Copper (Cu) is a trace metal and an essential component of several enzymes. Cu insufficiency is associated with often severe pathologic alterations including impairment of blood, liver, and immune systems [2, 3]. Cu ions stimulate endothelial cell proliferation [4] and migration [5] and modulate angiogenesis *in vivo* [6–8]. An extensive angiogenesis was a prominent feature around intra muscular (i.m.) implanted Cu disks [9].

On the other hand, corrosion of Cu implants with release of Cu ions may cause acute and chronic inflammation and in some cases even necrosis and sterile abscesses in tissues, including the brain [10–12], bone [13], abdomen and thorax and their organs [14–16], and muscle [17]. Cytotoxicity after Cu exposition has been studied mainly *in vitro* [18–23]. Cellular actions of Cu include destabilization of membranes via superoxide and hydroxyl radicals, affinity to RNA and DNA and inhibition of the transcription process [24, 25], inhibition of calmodulin, a regulator of, e.g. intracellular Ca^{2+} [26], and induction of apoptosis [27]. Excess Cu within cells after *in vivo* implantation has been found in, e.g. hepatocytes [15]. In Wilson's disease and Menke's syndrome the accumulation of Cu is associated with cytotoxic effects [28, 29]. The cytotoxicity of Cu has been used in cancer chemotherapy [30]. On the basis of the available literature on Ti and Cu these materials are associated with widely different biological outcomes. However, the early cellular responses at the implant-tissue interface *in vivo* have not been analyzed. The rat subcutaneous (s.c.) implantation model using the dorsum permits an analysis of cellular reactions as a function of time. Cells and molecules in the different compartments (material surface, exudate, and fibrous capsule) may be

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investigated quantitatively in response to alterations of implant material properties. Using this model of retrieval of exudate and implants the early cellular responses to implants with different surface chemical functionalizations have recently been examined [31, 32]. The aim with the present study was to investigate kinetics of the early cellular chemotaxis and cell viability around these two model surfaces and in sham sites by using this model.

2. Materials and methods

2.1. Implants

Circular implant Ti disks (diameter 10 mm; thickness 1 mm) were used. The disks were made by machining of commercially pure Ti (Grade 1). The surface roughness (Rq) was $< 0.2\,\mu m$. Half of the implants were covered with minimum thickness of 400 nm Cu by physical vapor deposition (PVD) in ultra high vacuum ($P \sim 10^{-7}$ torr). The Ti implants were sterilized in 70% ethanol solution for minimum 30 min and rinsed in sterile filtered Hank's balanced salt solution (HBSS; 0.7 mM PO_4, 0.15 M NaCl and $1\,g\,L^{-1}$ glucose supplemented with $0.1\,g\,L^{-1}$ Ca^{++}) before insertion. The Cu disks were dipped for 5 s in 70% ethanol solution and once in HBSS solution, respectively, before the insertion. A longer cleaning procedure resulted in Cu release from the surface. None of the implant types indicated endotoxins above the recommended level after the cleaning procedure (Limulus Amebocyte Lysate; < 9.5 EU/implant disk).

2.2. The animal model

The rat animal model has previously been described in detail [32]. Thirty-six female Sprague–Dawley rats (200–250 g) were fed on standard pellet diet and water. After intraperitoneal (i.p.) anaesthesia (2 : 1 : 1 solution of diazepam, 0.9% saline and sodium pentobarbital) the rats were shaved and cleaned with 5 mg/ml chlorohexidine in 70% ethanol) on the back. Each rat received four implants (2 Ti + 2 Cu) s.c. and in two operated sites no implants were inserted (sham). The wound edges were closed with two sutures (Ethilon 5-0, Ethicon®, Johnson & Johnson, Brussels, Belgium).

Explantation was done after 1, 3, 12, 18, 24, and 48 h (six rats at each time point). The rats were sacrificed by an i.p. overdose of sodium pentobarbital followed by immediate cutting of the sutures and gentle separation of the wound edges.

The implants were carefully removed from the pockets, rinsed once in sterile filtered HBSS and incubated at 37 °C (water bath) for 2 h in sterile HBSS supplemented with enzyme inhibitors (2 mM Pefabloc, Roche Diagnostics, Scandinavia AB, Bromma, Sweden; 4.2 μM Leupeptin and 0.3 μM Aprotinin, both from Sigma-Aldrich AB, Sweden). After the implant retrieval the exudate in the pocket was collected by repeated aspirations of totally 300 μl sterile HBSS (with enzyme inhibitors). The exudate was stored in polystyrene tubes at 4 °C until the end of the surgical procedure.

The animal procedure was approved by the Local Ethical Committee for Laboratory Animals (116/99).

2.3. DNA measurements, leukocyte enumeration and viability

DNA was analyzed for three types of cells: associated with implant surface, medium (cells detached from the implant during the *ex vivo* incubation) and the exudate (free cells retrieved with the exudate). The determination of DNA was performed as previously described [32] using the fluorescence marker Hoechst 33258 assay [33] detected in a luminescence spectrometer (Perkin-Elmer Ltd, Beaconsfield, Buckinghamshire, England) at excitation wavelength 360 nm and emission at 450 nm. The mean values ± SEM were calculated ($n = 5$ or 6 for exudate, $n = 10$ to 12 for medium, $n = 10$ to 12 for implants). The total number of polymorphonuclear granulocytes (PMN) and mononuclear cells and their relative proportions (%) in the exudate were counted by light microscopy using a Bürker chamber. Exudate cell viability was determined by Trypan blue dye exclusion. Mean numbers ± SEM was calculated ($n = 3$ to 6).

The content of lactate dehydrogenase (LDH) (an indirect parameter of cell lysis) was analyzed both in the exudate *in vivo* and in the incubation medium after the *ex vivo* implant incubation [34] (C-Laboratory, Sahlgrens University Hospital). The mean values (± SEM) were calculated ($n = 5$ to 6).

2.4. Statistics

Wilcoxon signed rank sum test and Mann–Whitney test (Wilcoxon two sample test) were used for the statistical analysis. The level of confidence was set to more than 95% (i.e. $p < 0.05$ is significant). No more than 10 tests of each variable were performed and no correction for mass significance was done. Significant differences are given in the legends of figures and tables.

3. Results

3.1. Cell recruitment and distribution

The inflammatory response, as judged either by measurement of the DNA content or enumeration of cells in exudate specimens, revealed three different types of kinetics (Fig. 1, Table I and Table II). The sham sites had a consistent, low amount of recruited cells throughout the 48 h observation period. Ti and Cu elicited an increased early influx of cells (up to 12 h) in comparison with sham operated sites. However, in contrast to sites with Ti, a marked increase in the number of inflammatory cells was detected at Cu. A persistent and high accumulation of cells was then prevalent around Cu throughout the observation period.

At early time periods (1–12 h) cells were equally distributed on the implant surface and in the exudate (Table I). At peak inflammation (18–24 h) the cells were distributed to a higher degree to implant surfaces than to surrounding exudates.

The analysis of inflammatory cells in the exudate revealed a higher proportion of mononuclear cells than PMNs (Table II). In sharp contrast, a markedly higher proportion of PMNs was detected around Cu at 12 h and onwards.

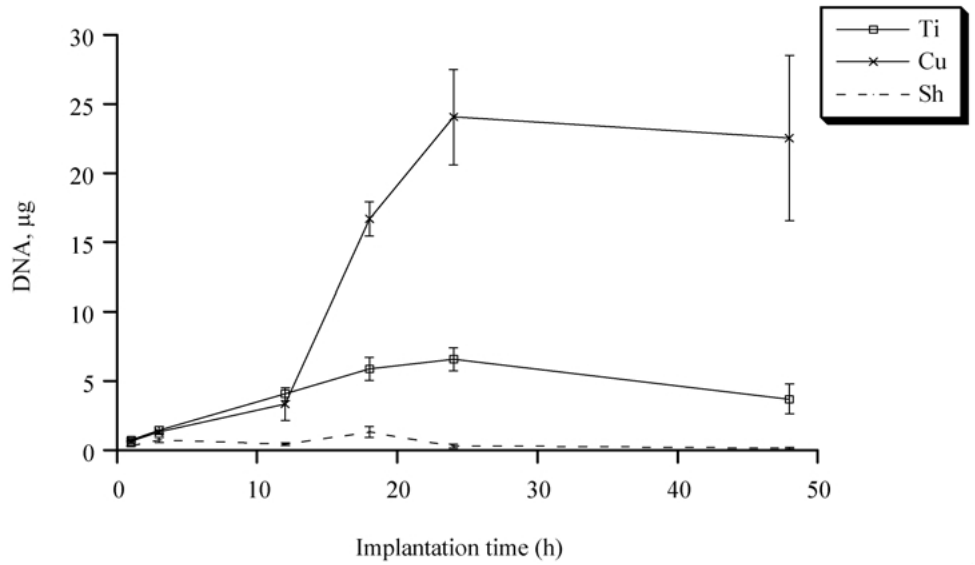

Figure 1 The total DNA content (µg) after 1, 3, 12, 18, 24 and 48 h in Ti, Cu, and sham sites. Mean ± SEM. Ti vs. Cu (18 h, 24 h, 48 h) $p < 0.05$, Ti vs. Sh (3 h, 12 h, 24 h, 48 h) $p < 0.05$, Cu vs. Sh (3 h, 12 h, 24 h, 48 h) $p < 0.05$.

3.2. Cell viability

Morphological analysis (using trypan blue dye exclusion) of Ti, Cu and sham exudate cell viability showed mean percentages between 75–95%, 72–89%, and 61–90%, respectively, during the experimental period (data not shown). No significant differences between the implant sites were found. A low amount of LDH (< 5 ukat/L) was detected around Ti, Cu, and in sham exudates during the initial 1 and 3 h. The total amount of LDH was markedly increased (six- to seven-fold) around Cu implants after 18–48 h in comparison with Ti and sham sites (Fig. 2). Ti and sham sites displayed similar LDH contents (below 5 ukat/L) during the experimental period (exception at 24 h with Ti having a significantly higher amount than sham).

The detected amounts of extracellular LDH during a 2 h *ex vivo* incubation of cells adherent to Ti and Cu were lower than those *in vivo* (Fig. 3). The levels were below 1 ukat/L at all times, except for Cu disks at 24 h (2.2 ukat/L). In contrast to the *in vivo* determinations, the LDH levels were higher around Ti than Cu during the initial 18 h observation period. Further, and also in contrast to the *in vivo* observations, after the rise of LDH at 24 h, a decrease was observed after 48 h around Cu.

TABLE I Amount of DNA (µg) in the exudate, from the implant surface and in the incubation medium after 2 h *ex vivo* incubation and total DNA content after 1, 3, 12, 18, 24, and 48 h. Mean ± SEM

Time	DNA (µg) (mean ± SEM)							
	Exudate			Implant + medium		Total		
	Ti	Cu	Sh	Ti	Cu	Ti	Cu	Sh
1	**0.4** ± 0.1	**0.2** ± 0.1	**0.3** ± 0.0	**0.3** ± 0.0	**0.5** ± 0.0	**0.8** ± 0.1	**0.6** ± 0.1	**0.3** ± 0.0
3	**0.7** ± 0.1	**0.9** ± 0.1	**0.7** ± 0.2	**0.4** ± 0.0	**0.4** ± 0.0	**1.4** ± 0.3	**1.3** ± 0.1	**0.7** ± 0.2
12	**2.2** ± 0.3	**2.3** ± 1.0	**0.4** ± 0.1	**1.9** ± 0.3	**1.2** ± 0.2	**4.1** ± 0.5	**3.3** ± 1.2	**0.4** ± 0.1
18	**1.5** ± 0.1	**7.3** ± 0.7	**1.3** ± 0.4	**4.2** ± 0.6	**8.2** ± 1.0	**5.9** ± 0.9	**16.7** ± 1.2	**1.3** ± 0.4
24	**1.0** ± 0.3	**5.8** ± 1.6	**0.3** ± 0.2	**6.0** ± 0.8	**17.2** ± 1.8	**6.6** ± 0.8	**24.1** ± 3.5	**0.3** ± 0.2
48	**0.7** ± 0.2	**5.6** ± 1.8	**0.1** ± 0.1	**3.2** ± 0.7	**17.0** ± 3.0	**3.7** ± 1.1	**22.5** ± 6.0	**0.1** ± 0.1

DNA (implant + medium) vs. DNA (exudate) around Ti (18 h, 24 h) $p < 0.05$.
DNA (implant + medium) vs. DNA (exudate) around Cu 24 h $p < 0.05$.

TABLE II Total number of inflammatory cells ($\times 10^4$ /ml) (mean ± SEM) and the percentage of mononuclear cells and PMN in the exudate from Ti, Cu and sham operated sites after 1, 3, 12, 18, 24 and 48 h implantation

Time (h)	Ti			Cu			Sh		
	No. of infl. cells	% mono	% poly	No. of infl. cells	% mono	% poly	No. of infl. cells	% mono	% poly
1	**0.5** ± 0.5	0	100	**2.8** ± 1.2	59	41	**1.3** ± 0.8	88	12
3	**5.1** ± 1.4	74	26	**3.1** ± 1.1	47	53	**7.7** ± 2.4	59	41
12	**78.3** ± 10.9	66	34	**176.3** ± 43.2	21	79	**20.7** ± 3.8	64	36
18	**176.3** ± 60.9	50	50	**760.4** ± 179.8	28	72	**30.7** ± 5.2	76	24
24	**28.7** ± 9.3	55	45	**1563.0** ± 825.2	29	71	**81.0** ± 43.6	45	55
48	**53.8** ± 15.8	64	36	**688.0** ± 196.3	14	86	**16.8** ± 7.7	69	31

% polymorphonuclear cells Cu vs. Ti (12 h, 18 h, 24 h and 48 h) $p < 0.05$.
% polymorphonuclear cells Cu vs. Sh (12 h, 18 h, 24 h and 48 h) $p < 0.05$.

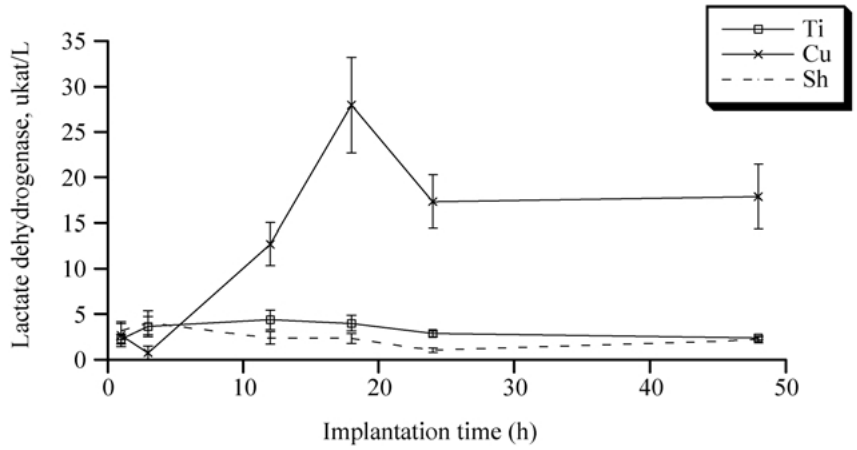

Figure 2 LDH content (ukat/L) in the exudate outside the implants after 1, 3, 12, 18, 24, and 48 h of s.c. implantation. Mean ± SEM. Ti vs. Cu (18 h, 24 h, 48 h) $p < 0.05$, Ti vs. Sh 24 h $p < 0.05$.

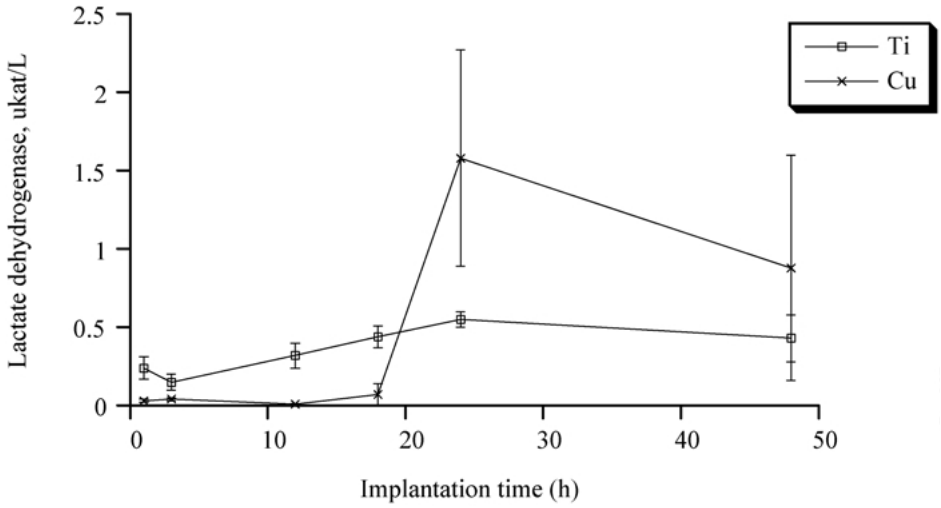

Figure 3 LDH content (ukat/L) in medium outside the implants after 2 h *ex vivo* incubation. Mean ± SEM. Ti vs. Cu 3 h $p < 0.02$, 18 h $p < 0.05$. Cu 1 h vs. 48 h $p < 0.01$.

4. Discussion

In this study the presence of implants, irrespective of material properties, elicited a greater chemotactic response than the surgical trauma *per se*. Further, a markedly higher number of inflammatory cells was recruited to the Cu interface in comparison with Ti and sham sites. Around Cu, but not Ti, this response was dominated by PMN. These findings suggest that the present *in vivo* model allows a discrimination between biocompatible and cytotoxic implant materials at very early time periods after implantation.

The proportion of different inflammatory cell types and the magnitude and kinetics of leukocyte accumulation around Ti was similar to observations for Au, CH3-Au and OH-Au implants after 3 and 24 h [32]. It was previously shown that Cu implants induce a pronounced and prolonged recruitment of inflammatory cells, as evaluated between 1 and 52 weeks after implantation [9, 16]. Cu implants in the peritoneal cavity of the rat elicited a large increase in the number of inflammatory cells and a relatively high proportion of PMN (32%) 1 week after implantation [16]. A persistent PMN containing exudate was also present after 10–52 weeks around i.m. implanted Cu [9]. Enzyme histochemistry revealed intense activity of PMN around Cu, especially around blood vessels [17].

The mechanism whereby Cu implants effectively induce the leukocyte and in particular PMN recruitment is not clear. One possible explanation is that Cu ions act as chemotactic factors. Previous studies *in vitro* would support this hypothesis, since Cu ions stimulate a proportion of the PMN population to assume a nonspherical, polarized shape, locomotion and chemotaxis (directed migration in a chemical gradient) rather than an augmentation of random movement (chemokinesis) [35, 36]. A possible mechanism for such a chemotactic effect of Cu is that Cu interacts with membrane receptors thereby mediating signal transduction leading to cell movement, exocytosis and respiratory burst. This possibility is indicated by the observation that Cu interacts with calcium binding motifs in the fibroblast growth factor (FGF) receptor-1 (FGFR-1), which is associated with increased FGFR-1 binding to heparin. Thus, Cu may play a role as a mediator of receptor–extracellular matrix interactions, of possible importance for cell migration and neovascularization [37].

Another possible mechanism for the recruitment of inflammatory cells to Cu implants is cell activation at the

tissue-material interface by stimuli present in the vicinity of the implant, leading to the secretion of chemotactic mediators. Hitherto, little information is available on the effects of Cu for the secretion of inflammatory mediators. However, *in vitro* studies have shown that Cu exposition is associated with increased PGE_2 release in human fibroblast-keratinocyte co-cultures [38]. A modulatory role of Cu for PGE_2 has been suggested, since Cu facilitates PGE_2 stimulation of luteinizing hormone releasing hormone (LHRH) release, possibly via the induction of disulfide bonds on the receptor/plasma membrane [39, 40]. Also, human fibroblast-keratinocyte co-cultures exposed to Cu responded with higher interleukin-6 release than controls [41].

In contrast to observations around Cu implants, in the present study the inflammatory response around Ti was markedly less intensive. This is in agreement with previous observations in different implantation models in soft and hard tissues (reviewed in Holgers *et al*. [1] and Brånemark *et al*. [42]).

Of particular importance in this context is the mechanism whereby Ti stimulates a transient recruitment of inflammatory cells and allows the repair/regenerative processes to proceed (leading to fibrous capsule formation in soft tissue and osseointegration in bone). The implantation of a Ti chamber *in vivo* caused PMN accumulation and an increase in leukotriene B_4(LTB_4) levels [43]. This finding together with observations *in vitro* that interactions between monocytes and Ti surfaces lead to secretion of cytokines [44] suggest that at least a partial explanation for the recruitment of cells to the Ti surface is due to the activation of inflammatory cells by the (protein-coated) Ti surface, and subsequent secretion of chemotactic factors. The effects of implantation of Ti and Cu on cytokine secretion *in vivo* is therefore of interest and the subject of a second part of this experimental study [45].

The present model allowed us to study the kinetics of cell viability at implant surfaces during *in vivo* conditions. Extracellular LDH is a marker of plasma membrane injury [18, 20, 46, 47] and is increased for example when monocytes are exposed to high concentrations of particulate materials *in vitro* [48]. Very high levels of extracellular LDH were detected in the *in vivo* exudates around Cu implants after 12 h. The high levels persisted throughout the observation period. In contrast, Ti and sham sites displayed essentially similar LDH levels and kinetics. On the other hand, the trypan blue dye exclusion data showed that the proportion of viable cells was not significantly lower around Cu than at Ti and sham sites. It is therefore likely that the high and persistent LDH levels around Cu were strongly related to the marked increase in the number of recruited inflammatory cells and subsequently to a higher total number of less viable cells at Cu than at Ti and sham sites. An additional explanation may be that also cells that were more distant to the Cu surface were negatively affected by Cu ions (e.g. cells residing in and/or migrating to the surrounding tissue). The *ex vivo* LDH data, quite surprisingly, did not indicate an early toxic effect of Cu on adherent cells (for implantation periods up to 18 h). Further, at the 48 h endpoint of this study there were no significant differences in the LDH levels between Cu and Ti *ex vivo*. Human THP-1 and mouse P338D1 macrophages reacted adversely *in vitro* to metal ions at similar concentrations as other cell types found in the oral cavity although different kinetics in LDH release, mitochondrial metabolic and protein synthesis were found depending on the type of metal ion and the species of the macrophage [21]. Previous studies on *in vitro* cytotoxicity induced by Cu and corrosion products released from Cu alloys have shown a reduced viability of human fibroblasts, keratinocytes, T-cells and B-cells [19, 41] and loss of membrane integrity of different cell lines [18, 20, 21]. In addition, Cu induced apoptosis in mouse pro-B cell line BA/F3b, involving expression of Bax, generation of reactive oxygen species and repression of NFκB [27]. In order to examine the mechanisms for metal-induced cell death, further studies are required on apoptosis and necrosis patterns during the initial response at the implanted surfaces. Of particular importance is then to relate the cellular viability and secretion of inflammatory mediators in the different ''compartments'' (material surface, exudate, fibrous tissue) to concentrations and possible ion gradients.

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