Expression of heat shock protein 70 (HSP70) at the interface of polymer–implants *in vivo*

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Synthetic polymer meshes are widely applied in the modern surgical approach for repairing abdominal wall defects. The implanted material is often observed leading to post-operative complications such as deficient abdominal wall mobility and adhesion formation with the abdominal cavity and/or abdominal organs. However, the functioning of the implant is primarily affected by the wound healing process guided by inflammatory events occurring at the tissue–material interface. This could presumably be influenced by the physicochemical properties of the polymer. With regard to it, the cellular and molecular processes involved in the successful restoration of the abdominal wall function are poorly understood. The present *in vivo* study, therefore, exemplary investigated in a rat model, the commercially available polymer-meshes Prolene® (polypropylene, PP), Mersilene® (polyester, PE) and Vicryl® (polyglactin 910), as well as new mesh variants consisting either of PP (EB) or a combination of PP and polyglactin 910 (A plus or Vypro®). The implanted material was evaluated by light and electron microscopy, immunohistochemistry as well as morphometry over an implantation period of 90 days. The data show that polymers induce heat shock protein (HSP)70, and its expression at the interface correlates inversely with the activity of the inflammatory reaction *in vivo*. Further, an ascent in HSP70 expression parallels the increasing implantation period and evolving foreign-body granulomas. Accordingly, a major role for HSP70 in modulating the local acceptance of polymers and as an additional marker for *in vivo* testing of polymers is suggestive.

1. Introduction

From the point of view that synthetic polymers behave like a foreign body *in vivo*, it can be assumed that their interaction with the recipient tissues exhibits an unphysiological stress. The histological features characterizing this stress condition *in vivo* are generally defined by the composition of the inflammatory cells, connective tissue formation and angiogenesis. These parameters are largely exploited to describe the biocompatible function of the polymers. However, all these *in situ* observations reflecting the *in vivo* condition may account for the stress response mounted by the cells in contact with the polymer and/or may be the result of protective cellular response to a polymer-specific stress.

The molecular rationale responsible for the protective cellular response to stress is assigned by the expression of a large number of intracellular stress proteins with pleiotropic functions for cellular homeostasis. For example, heat shock response (HSR) represents a cellular stress response that occurs following brief exposure of cells to supraphysiologic levels of heat [1, 2]. As an acute stress response, HSR is characterized by reversible cellular changes at the cell metabolism level, which allows the cell to adapt to unphysiological conditions. In general, the HSR in all organisms is a rapid and almost exclusive synthesis of a small number of intracellular proteins, the so-called stress proteins, including the heat shock proteins (HSPs), which are subdivided into families categorized by their apparent molecular weights. The most common members of the HSP family described so far are, among others, HSP25, 27, 60, 72, 73 and 90. The HSP70 family, a heterogeneous group comprised of both constitutively and inducible expressed stress proteins (HSC70/73 and HSP72), is the most abundant HSP in many organisms [3]. The fact that HSP72 is highly stress inducible led to the HSP70 family being the most extensively investigated HSP reported in the literature.

Taking into account that rapid synthesis of HSPs, particularly HSP70, enables the cell to tolerate stresses than might otherwise be lethal, this defence mechanism is found to be initiated by miscellaneous injurious agents including ischemia, heavy metals, such as arsenite and zinc, proinflammatory mediators, like TNFα and IL-1,

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and, classically, hyperthermia as well as LPS [4–7]. Furthermore, HSPs appear to play a particularly important role in the immune system, e.g. the expression of inducible HSP70 and HSP90 can be detected during antigen processing and presentation [8], during the differentiation process of the monocytic cell line U937 [9], as well as by mitogen activated T cells [10].

Though studies demonstrating that polymers exhibit the potential to induce HSR are lacking, the results obtained from such investigations might be significant in evaluating the biocompatible function of the material *in vivo*. Hence, the present study attempts to determine whether HSP70 is expressed at the interface of implanted polymers. For this purpose, surgical polymer-meshes were implanted into a standardized rat model. Immunohistological and morphometric analyses were performed to examine the expression of HSP70, which finally is correlated to *in vivo* representative parameters, such as the inflammatory infiltrate and/or connective tissue formation. It is shown that polymers are capable of inducing HSP70. Furthermore, a rise in the expression of HSP70 is visible by the increase in implantation time and foreign-body granuloma formation as well as by the low inflammatory reaction. In conjuncture to it, these results support the opinion that HSP70 besides being an *in vivo* marker for the biocompatibility of polymers, may play a decisive role in affecting the *in vivo* functioning of polymers.

2. Materials and methods

2.1. Experimental animals

One hundred male Wistar rats (250–300 g) were studied. The animals were housed under conditions of constant light and temperature and received a complete diet of rat feed *ad libidum* throughout the entire study, which was performed according to the rules of the ''Deutsche Tierschutzgesetz'' (AZ 23.203.2 AC 18, 17/94) and to the NIH guidelines for the use of laboratory animals. The animals were randomly divided into five groups (each $n = 20$).

2.2. Mesh modifications

The prosthetic materials used were polypropylene (PP), polyester (PE) and polyglactin 910 in different modifications and combinations. These permanent or absorbable polymers were manufactured as mono- or multifilament meshes with varying counts of yarn ($g\,1000\,m^{-1}$), weights ($g\,m^{-2}$), proportion of pores (%) and mechanical properties. The mesh-modifications were compared with current commercially available PP-mesh Prolene® (Table I).

2.3. Surgical procedure

Anaesthesia was achieved with a mixture of ketamine hydrochloride ($80\,mg\,kg^{-1}$ intraperitoneal, i.p.) and xylazine ($8\,mg\,kg^{-1}$ i.p.). The skin was shaved and disinfected with Betaisodonna® solution. After midline incision, a full-thickness defect was performed resecting the rectus muscles with the peritoneum (except for the skin), 2 cm distal of the xiphoid, on an area 2×3 cm *en bloc*. Each mesh (2×3 cm) was fixed in an inlay position continuously with Prolene® 5/0 without overlap between muscles and prosthesis. Skin closure was finally obtained with silk 3/0 continuous sutures. No antibiotic treatment was given before or during the experiment. Sham controls were performed by simple closure of the midline laparotomy by continuous Prolene® 5/0. A separate control group received no operation.

2.4. Observation period

From each group ($n = 20$) five animals were sacrificed at three, seven, 14, 21 and 90 days after implantation of the meshes. Through the whole observation period of 90 days all animals of each group were daily controlled to objective local and systemic complications.

2.5. Antibodies

The antibodies used included polyclonal rabbit anti-heat shock protein 70 A500; 1 : 200 (Dako, Hamburg, FRG) and monoclonal anti-HSP70/HSC70 SPA-820; 1 : 200 (Biomol, Hamburg, FRG). Anti-HSP70 reacts strongly with the two major HSP70s (HSP72 and HSP73).

2.6. Morphological study

Specimens were studied by light (LM) and transmission electron microscopy (TEM). For LM, tissue samples were fixed in 10% formalin, embedded in paraffin, and sections were stained with haematoxylin and eosin (H&E), as well as periodic-acid Schiff (PAS) plus diastase and Elastica van Gieson (EvG). For TEM, tissue specimens were fixed in 3% cacodylate-buffered glutaraldehyde for 30 min. After post-fixation in osmium, buffered in 0.1 M cacodylate, they were dehydrated in ethanol and embedded in epon. Semi-thin-sections (1.0 μm) were stained with methylene blue-azure II. Ultra-thin-sections were mounted on copper grids, stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (Philips, EM400T).

TABLE I Main data of the mesh-modifications used in this study, showing the main properties of each mesh variant

	Prolene®	Mersilene®	EB	A plus	Vicryl®
Material	Polypropylene	Polyester	Polypropylene	Polypropylene polyglactin 910	Polyglactin 910
Type of filament	Monofilament	Multifilament	Multifilament	Multifilament	Multifilament
Weight $g\,m^{-2}$	108.5	39.5	70.5	54.65	53.6
Pores%	83.52	89.78	82.42	90.77	–

2.7. Immunohistochemistry

Immunohistochemistry was performed at the paraffin embedded material using the avidin–biotin complex method, with diaminobenzidine as the chromogen. The same staining methodology was used in all tests and control animals.

2.8. Morphometry

Morphometric evaluation consists of quantitative cell analysis of the inflammatory and soft-tissue reactions. Morphometry is performed in the center and in the suture zone of the mesh. The cells were counted each in five H&E-slides in ten fields, at a grid of ten points (× 140, area 0.106 mm^2), and at the interface (0–300 μm; area, 636 μm^2) during TEM. The parameters measured were the inflammatory infiltrate (partial volume, PV,%), the connective tissue (PV%), macrophages (%), granulocytes (%), lymphocytes (%) and HSP70-positive cells (%).

2.9. Statistics

Statistical analysis was carried out using the *Statistical Package for Social Sciences* (SPSS) software. All functional and morphological results were analyzed for statistical significance using a corrected analysis of variance, i.e. least-significant-differences (LSD) test according to Bonferroni, followed by independent *t*-tests when significant differences (versus Prolene®) were indicated. p values < 0.05 were considered to be significant.

3. Results

3.1. Prolene®

The cell-infiltrate in contact with these meshes consists of a heterogeneous population of cells mainly polymorphonuclear granulocytes (PMNs), macrophages, a few eosinophils and lymphocytes. Plasma cells were scarcely observed. The partial volume (PV) of the inflammatory constituent revealed a phasic appearance. It was highest at day 3 (28.3%), and finally decreased to levels of about 16% at day 7 and 14. At day 21 an increase could again be detected (31.5%), which showed a reduction to 21.1% at day 90 (Fig. 1a). In the early post-implantation period, macrophages compared to PMNs constituted the predominating inflammatory cell type (Fig. 1c, d). While PMNs in the course of the implantation period, except at day 21, showed a decreasing density in the interface, macrophages revealed a rising accumulation rate and were the major inflammatory cell type after 90 days (Fig. 1c, d). Moreover, macrophages were observed transforming to epitheloid-like cells from day 14 onwards, and, in parallel, with a continuously large number of multinucleated giant cells in the contact zone of the monofile mesh fibers.

Additionally, other inflammatory cell types, such as lymphocytes (Fig. 1e) and plasma cells, could only be detected in peripheral granuloma regions, yet were in small numbers in contrast to eosinophils, which were visible in the inner zone of the interface mainly in direct contact with the mesh fibers.

Furthermore, the PV of the connective tissue in the $< 300\,\mu m$ interface showed no connective tissue formation at day 3, whereas at day 7 a PV of 46.81% could be seen. In the following days of experimentation, the PV of the connective tissue reached a maximum of merely 54.1% at day 90 (Fig. 1b).

3.2. EB

The tissue reaction of this PP-mesh modification, EB, was different to that of the Prolene®-mesh described above. EB displayed at day 3 and 7 a significantly increased inflammatory reaction ($P < 0.001$; Fig. 1a, c, d) with a massive acute purulent appearance localized to the direct interface of the mesh filaments and recipient tissues. At days 14–90 the latter was seen involving the whole artificial abdominal wall, comprising skin and abdominal organs, with strong adhesion formation. Furthermore, there was a marked oedematous stroma reaction with subsequent thickening of the artificial abdominal wall, final wound edge separations and bacterial superinfection were obvious. The morphology was characteristic of an extensive fibrinoid and necrotic inflammation with formation of local (micro)-abscesses in the recipient tissues (Fig. 2a).

Other inflammatory cells, such as lymphocytes and plasma cells, were seen in phasic appearance at day 7 and with a maximum at day 21, which at day 90 showed its minimum extent.

PV examination of the connective tissue revealed enhancing connective tissue formation (Fig. 1b), demonstrating a strong fibrous tissue reaction guided by EB. Further, TEM analysis showed a disturbed collagen-neogenesis, e.g. degenerated collagen bundles in the extracellular matrix could be recognized.

3.3. A plus

This is a reduced PP mesh combined with polyglactin 910 exhibiting a significantly reduced inflammatory reaction compared to Prolene and EB ($P < 0.01$; Fig. 1a). The interface showed macrophages as the major inflammatory cell types (Fig. 1c), whereas PMNs were found in significantly decreased number ($P < 0.001$; Figs 1d and 2b). After 90 days the tissue reaction was seen to be defined by granulomas of foreign-body type containing a moderate number of giant cells. Moreover, a significantly increased number of lymphocytes as inflammatory cells were detected ($P < 0.01$; Fig. 1e). Additionally, formation of necrosis was absent, and the amount of connective tissue formed around the mesh fibers was significantly reduced compared to EB, Prolene® and Vicryl® (Fig. 1b).

3.4. Mersilene®

During the first 14 days of implantation the polyester mesh Mersilene showed the lowest inflammatory reaction, which at 90 days displayed a lower PV of inflammatory cells in the interface (this was only otherwise shown by the Vicryl® mesh, Fig. 1a).

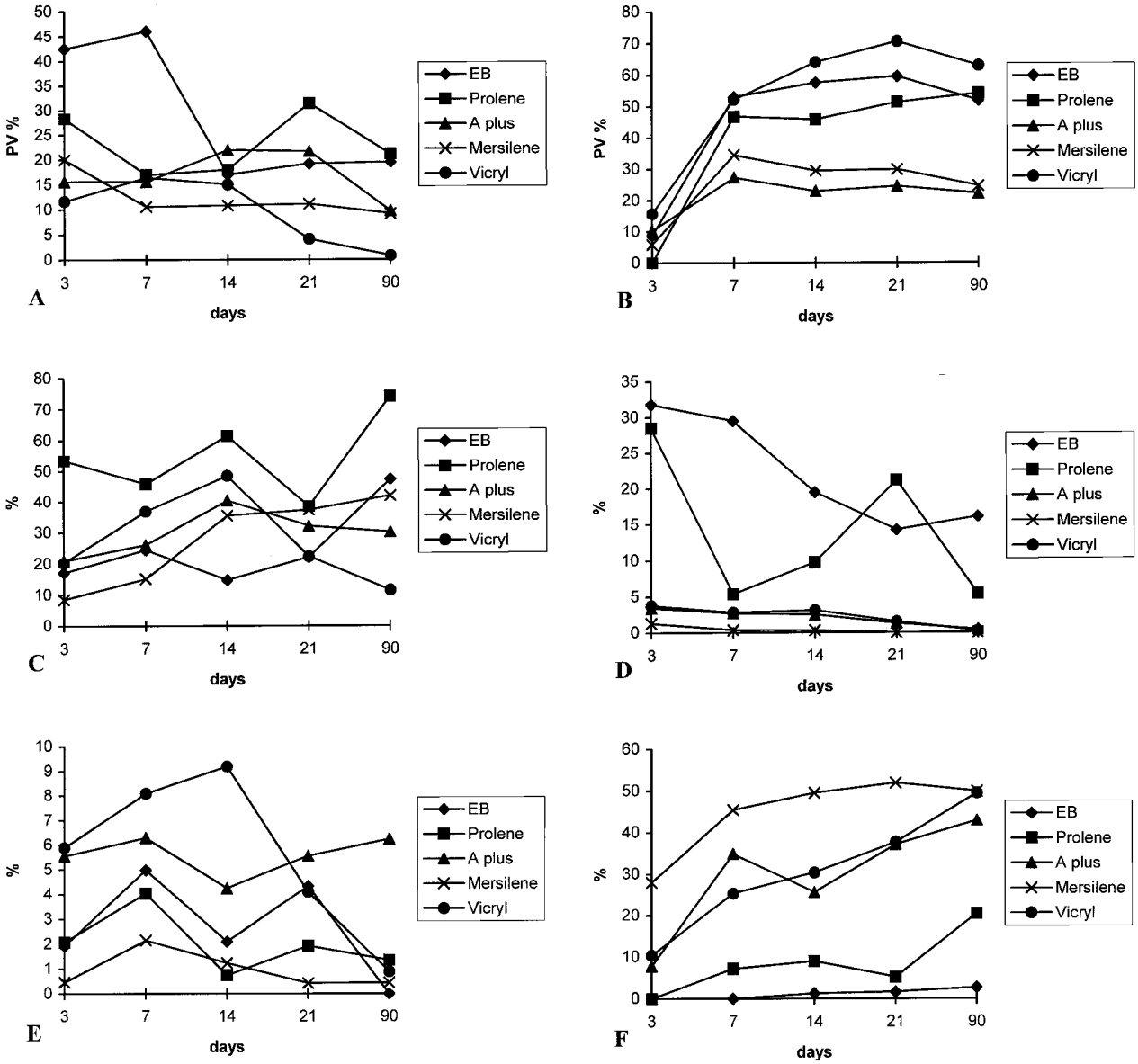

Figure 1 Morphometric data of the interface mesh–polymer-fiber (< 300 μm): (A) partial volume (PV) of the inflammatory infiltrate (%), (B) PV of connective tissue formation (%), (C) macrophages (%), (D) polymorphonuclear neutrophils (PMNs;%), (E) lymphocytes (%) and (F) HSP70-positive cells (%).

However, local reaction was characterized by immigrating macrophages (Fig. 1c), which seemed to mature to epitheloid cells within the first two or three weeks and to form a large number of multinucleated giant cells. Generally, the Mersilene mesh induced a distinct granulomatous reaction, e.g. PMNs were seen to be nearly absent (Figs 1d and 2c). The PV of the connective tissue revealed an increase compared with that of the A plus mesh and thus showed the lowest deposition of collagen bundles at the interface (Fig. 1b). The number of lymphocytes was the lowest compared with those in all meshes investigated (Fig. 1e).

3.5. Vicryl®

The absorbable mesh modification Vicryl® showed a macrophage-dominated tissue reaction within the first 14 days of the experiment (Fig. 1c). The number of PMNs was low during the whole examination period (Fig. 1d). After 14 days the macrophages declined to the lowest levels, yet the levels were higher than those of PMNs observed in this study (Fig. 1c). Further, in contrast to the similar amount of infiltrated lymphocytes detected compared with the A plus mesh containing polyglactin 910 described above (Fig. 1e), the formation of connective tissue was notably highest after Vicryl® implantation (Fig. 1b). Additionally, after 90 days, the Vicryl® mesh was found to be incompletely absorbed.

3.6. HSP70 expression

The HSP70 expression detected was significantly elevated in the Vicryl®, Mersilene® and A plus mesh modifications compared to EB and Prolene® (Figs 1f and 2a–f). The highest levels, however, could be found in Mersilene®, predominantly showing macrophages and a distinct epitheloid foreign-body reaction yet with the lowest number of PMNs in the interface. In contrast, with increasing numbers of PMNs the interface showed lower levels of HSP70 expression, namely in macrophages and

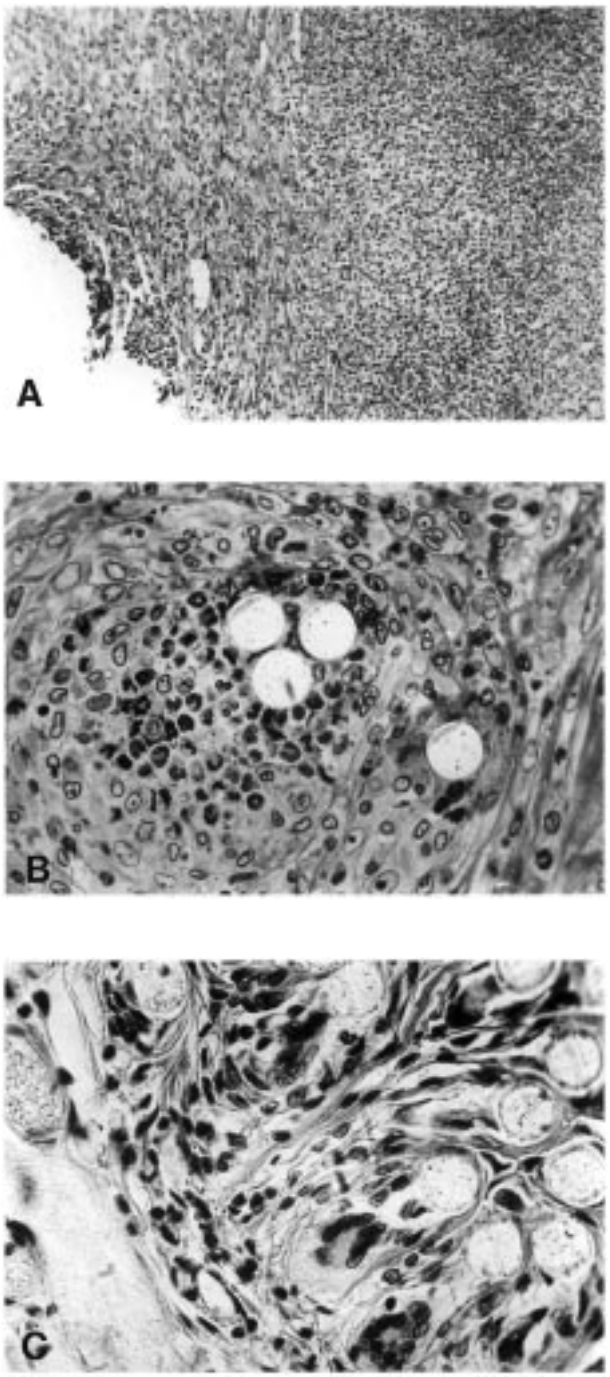

Figure 2 (A) Suppurative inflammation in the interface of the EB mesh; day 90, H&E, × 80. (B) Subacute inflammatory reaction with evidence of PMNs and macrophages in the interface of the A plus mesh; day 14, H&E, × 250. (C) Chronic granulomatous inflammation with multinucleated giant cells; Mersilene®, day 90, H&E, × 250.

giant cells. Moreover, HSP70 expression was found to increase with time over the course of implantation (Fig. 1f), with the macrophages transforming to epitheloid cells and foreign-body granulomas.

4. Discussion

The present *in vivo* study in a rat model demonstrates that different implanted synthetic polymer meshes exhibit different potential to evoke an inflammatory reaction, yet with varying degrees of acute, chronic inflammatory response and fibrous tissue formation. Other studies have reported that the weight, fabric and chemical composition of the polymer may affect the specific mode of cell activation in the inflammatory response of the host tissue. In this recent study, though, it could be observed that implanted meshes with different chemical compositions but similar fabrics (the multifile A plus, Mersilene® and Vicryl®) may generate a similar chronic inflammatory response, which showed predominantly as macrophages and in their transformation to epitheliod and giant cells. Yet, the other multifile EB set caused acute inflammation populated densely by PMNs, monocytes/macrophages and other inflammatory cells resulting in massive necrosis. In contrast to it, the monofile and high weight Prolene® mesh provoked a more chronic inflammatory response, which was comparable to that shown by the other aforementioned meshes. Further, the type and degree of occurrence of inflammatory cells could specify the mode of this inflammatory response, e.g. the appearance of PMNs during the long implantation period of Prolene® is suggestive of an active inflammatory reaction. The opinion that different chemical compositions of polymer might also be responsible for the increased accumulation of certain types of inflammatory cells is apparent from the Vicryl® mesh compared to its related A plus mesh implant showing a large number of macrophages, which is indicative of enhanced emigration and phagocytic activity of these cells resorbing this material. Additionally, following injury and/or implantation the wound healing process is generally accompanied by connective tissue formation, which coincides with the results presented in this study, though occurring to varying extents depending upon the polymer applied. However, the two different polymer meshes, Mersilene® and A plus, having similar fabrics, demonstrated a comparable low degree of fibrosis and thus support the view that other endogenous factors and mediators may be involved in this process of fibrous encapsulation of implants. Accordingly, it can be inferred that inflammatory reaction progressing to a chronic inflammatory response and fibrosis might be misleading with regard to biocompatibility studies and the inflammatory response to implants.

Implantation of foreign synthetic material into the living body might cause a stress condition, which could be responsible for promoting molecular mechanisms playing a regulatory role in wound healing and in the integration processes of the implant. In this context, to encounter adverse environmental stress the living cellular system might drive a repertoire of stress responses including the phyllogenitically oldest one, the HSR. This response is characterized by the induction of heat shock proteins, in particular HSP70.

The findings of this study demonstrate an inverse correlation between the range of HSP70 and the degree of inflammation, and implicate that polymers are capable of inducing HSR by recipient tissue cells. However, the level of HSP70 detected indicates that different polymers are capable of inducing this protein to different extents. The aforementioned polymer-meshes, promoting a chronic, macrophage-dominated inflammatory reaction, appear potentially to induce this stress response protein in contrast to other mesh-modifications, which drive acute inflammation highly populated by PMNs and show significantly decreased HSP70-positive macrophage colonies at their interface. Regarding the highly

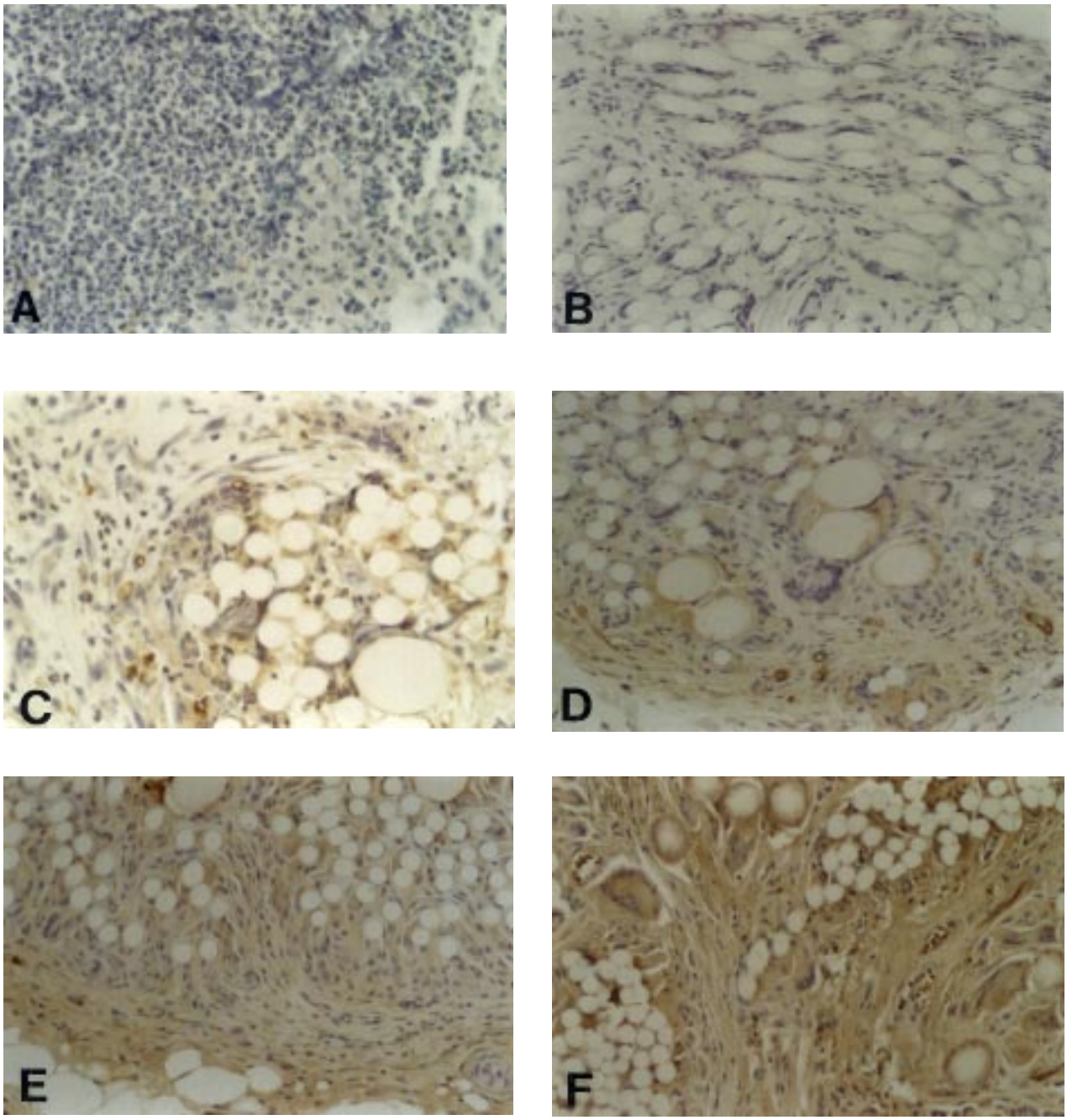

Figure 3 HSP70 expression in the interface of mesh–polymer-fiber demonstrated at the A plus mesh, Prolene® and EB: (A) locus of suppurative inflammation without HSP70 expression (Prolene®; day 7; × 250); (B) persistence of an acute inflammatory component (PMN) is associated with a low HSP70 expression at the interface (EB; day 90; × 100); (C)–(F) increasing HSP70 expression at the interface of the A plus mesh, a composite mesh-modification of multifile polypropylene and polyglactin 910. (C) day 7, × 250; (D) day 14, × 100; (E) day 21, × 100; (F) day 90, × 100); note that HSP70 expression rises with the maturation of the epitheloid cell granuloma.

recognized role of monocytes/macrophages in affecting the wound healing process, these observations support the view that immediate early inflammatory events initiated after implantation of the polymers might be responsible for increased recruitment of monocytes/macrophages into the implant and thus affecting its outcome by modulating the progressing inflammation to chronic and/or acute inflammatory reaction. Following this opinion, the marked increase in HSP70 could be the result of the protective and regulatory mechanisms occurring to maintain cellular homeostasis, which corroborates with the known role of HSP70 in cell protection and repair of cell damage caused by various stressors [4, 7, 11, 12].

Moreover, recent *in vitro* studies support the idea, showing that stressed peritoneal and alveolar macrophages produce decreased amounts of TNF in response to LPS, and human monocytes exhibit marked reductions in LPS-stimulated TNF and IL-1 production when these cells are first subjected to stress [13–19]. Ensor and coworkers in 1994 [20] compared the effects of elevated temperatures within the usual fibril range on the expression of TNF and IL-6 *in vitro* in LPS-stimulated human macrophages derived from peripheral blood monocytes. Hyperthermia differentially affects the biosynthesis of these proinflammatory cytokines by almost complete inhibition of TNF-synthesis in human, LPS-stimulated macrophages at 40 °C, while the release of IL-6 in these cells was preserved. Despite this, these cytokines as well as their inducer LPS are well known

factors for inducing apoptosis. HSP70 has recently been shown to protect cells from apoptotic damage [12]. Accordingly, in mesh implants, e.g. Mersilene®, showing the highest HSP70 content, a low apoptotic cells rate was also observable (own data to be published), which coincides with the reported protective role of HSP70. Whether the abovementioned immunomodulatory effects of HSPs are responsible for inflammatory reaction after polymer implantation, remains to be unveiled. However, the monocytes matured to macrophages after contact with different polymers expressing varying levels of HSP70, and the levels of HSP70 detected show a reversable relationship with TNFα liberation: are both suggestive of the immune response modulating activity of HSP70 (manuscript in preparation).

In addition to the general protective and immunomodulatory role of HSPs as molecular chaperones by protein denaturation during stress, their role in the inhibition of gene expression of proinflammatory cytokines is also well recognized. Recently, it was demonstrated that heat shock response inhibits inducible nitric oxide synthetase (iNOS) gene expression [21]. iNOS is responsible for the sustained production of nitric oxide (NO), which is a recognized pathophysiological mediator of oxygen radical production. Oxygen radicals, however, can play a pivotal role in mediating polymer-associated tissue damage. The presence as well as the modulation of iNOS was not investigated in this study, yet the production of NO has been reported by others studing synthetic polymers.

Altogether, the recent study assumes that HSP70 may have an eminent role in polymer-associated inflammatory reaction. The known immunomodulatory and homeostasis regulatory effects of HSP70 to down-regulate proinflammatory cytokines and cellular damage could be crucial in determining whether a polymer induces an acute or a chronic inflammation at the interface to the host–tissues. Besides, HSP70 could be an additional *in vivo* marker for the local acceptance of polymers.

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