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Journal of Immunological Methods 328 (2007) 118–127

JIM
Journal of
Immunological Methods

www.elsevier.com/locate/jim

Research paper

Simultaneous analysis of multiple serum proteins adhering to the surface of medical grade polydimethylsiloxane elastomers

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Received 19 February 2007; received in revised form 24 July 2007; accepted 29 August 2007
Available online 19 September 2007

Abstract

Although polydimethylsiloxane (PDMS, silicone) elastomers are presumed to be chemically inert and of negligible toxicity, they induce a prompt acute inflammatory response with subsequent fibrotic reactions. Since local inflammatory and fibrotic side effects are associated with the proteinaceous film on the surface of silicone implants, the process of protein adherence to silicone is of practical medical relevance, and interesting from theoretical, clinical and biotechnological perspectives. It is hypothesized that the systemic side effects resembling rheumatoid and other connective tissue diseases may be triggered by local immunological changes, but this functional relationship has yet to be defined. Because the proteinaceous film on the surface of silicone has been identified as a key player in the activation of host defense mechanisms, we propose a test system based on a proteomics screen to simultaneously identify proteins adsorbed from serum to the surface of silicone. Herein, we describe protein adsorption kinetics on the surface of silicone implants, correlate the adhesion properties of serum proteins with the occurrence of adverse reactions to silicone, and successfully discriminate their signature on the silicone surface in a blinded study of patients suffering from fibrotic reactions (as determined by Baker scale) to silicone implants.

Keywords: Polydimethylsiloxane; Protein adsorption; Fibronectin; Silicone implants; Biocompatibility; *In vitro* test

1. Introduction

Polydimethylsiloxane elastomers are used for the production of numerous active and passive implantation devices that are in direct, and sometimes prolonged, contact with human tissues. Routine medical practice during the last decades has endorsed silicone as the most widely used implant material despite the occurrence of intermittent local and systemic adverse immunological effects. Local side effects generally include formation of

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0022-1759/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jim.2007.08.016

a hypertrophic fibrotic capsule around the implant that is prone to contraction in later stages, causing severe pain, local tissue damage and implant function impairment (Siggelkow et al., 2003; Vasey et al., 2003). Although meta-analyses failed to provide a definitive correlation between silicone implants and increased risk of autoimmune diseases, various autoimmune-like syndromes in patients with silicone implants have been recurrently reported in women with silicone mammary implants (SMIs) (Appleton and Lee, 1998; Smalley et al., 1996; Iyoda et al., 2005) and in patients receiving other silicone and silicone-coated medical devices (Kinnari et al., 2003; Goldblum et al., 1992; Yammine et al., 2005).

A thin proteinaceous film on the implanted silicone surface is acquired immediately after the implantation and is a key activator of the immune response (Elwing, 1998; Hu et al., 2001; Peltroche-Llacsahuanga et al., 2001). Numerous proteins involved in host defense, structural/extracellular matrix proteins, transport proteins, and stress proteins have been found to be deposited on the surface of medical grade silicones and other biomaterials, both *in vivo* and *in vitro* (Wagner et al., 2003; Backovic et al., 2007). The exact mechanisms by which proteins trigger immune responses remain to be clarified, but some of the adhering proteins expose cryptic antigens that may activate macrophages, which, in turn, recruit lymphoid cells and induce trans-differentiation of fibroblasts to myofibroblasts in the surrounding tissue (Tang and Eaton, 1993; Workalemahu et al., 2003; Shen et al., 2004). Chronic inflammation and myofibroblast proliferation often results in dense collagenous fibrosis shortly after implantation (Siggelkow et al., 2004). Furthermore, peri-implant connective tissue capsules contain a population of dendritic cells (Wolfram et al., 2004), providing a prerequisite for *in situ* initiation of an immune reaction. Since the proteins adhering to silicone directly challenge the host response, we examined the initial proteinaceous film formation. Modeling of such a system encompasses numerous factors that strongly influence proteinaceous film formation. The complex interactions of conformational protein changes, flow conditions, competitive adsorption, initial stages of blood clotting and complement activation on protein film formation, a phenomenon of protein displacement known as the "Vroman effect", have been detailed by other groups and will not be discussed here (Vroman et al., 1980; Turbill et al., 1996; Ortega-Vinuesa et al., 1998; Wagner et al., 2003). However, although some of the proteins adhering to silicone have been described as modulators of host defense, the systemic effects of the complex proteinaceous film on the silicone-tissue interface remain poorly understood (Hocking and Kowalski, 2002; Kim et al., 2005; Gallagher et al., 2006; Shen et al., 2004).

To examine the possibility that serum protein adhesion to silicone correlates with development of adverse effects, standardized silicone surfaces approved for human implantation were incubated with serum and the adhering proteins were analyzed at different time points (Ma and Dickinson, 2004; Coombes et al., 2001). For this purpose, a silicone-linked immuno-sorbent assay (SILISA) was constructed as a simple test system that allows simultaneous determination of protein adhesion patterns in different patients. The proteins selected for the SILISA were adapted from numerous reports, including our own, on protein deposition to polydimethylsiloxane elastomers. From the many proteins reported to bind to the surface of silicone, we initially tested 30 known to play a prominent role in the activation of innate and adaptive host defense systems and/or initiation of autoimmune phenomena. Correlating their adhesion with the fibrosis status of patients, we excluded 23 proteins that had p-values smaller than 0.05 between the absorption values in fibrotic patients compared to unaffected controls. In a cohort study, this test system distinguished patients with fibrosis and controls that did not develop fibrosis in response to implanted silicone material.

Our model represents clear progress towards better understanding the molecular mechanisms behind the adverse reactions to silicone, and we believe our results will provide the basis for defining parameters for prevention and treatment of silicone implant side effects.

2. Methods

2.1. Study design

Seventy women undergoing cosmetic breast augmentation were separated into two groups according to the Baker score of breast connective tissue fibrosis (Kamel et al., 2001): patients with (Baker score ≥ 3; $N=28$; median age 51 years), and without extensive fibrosis (Baker score <3; $N=42$; median age 47 years). All patients gave informed consent and the study was approved by the Ethical Committee of the Innsbruck Medical University (Study Number AN2218). Sera collected from the patients were immediately aliquoted, blinded, coded, and frozen at −80 °C until use.

2.2. Chemicals/reagents

If not stated otherwise, chemicals were purchased from Sigma-Aldrich (Vienna, Austria). Proteins were

eluted from silicone either in 2% SDS, or in urea buffer (consisting of 7 M urea, 2 M thiourea (both from Calbiochem, Bad Soden, Germany), 30 mM TRIS, 4% CHAPS, pH 8.5). A protease inhibitor cocktail composed of pepstatin, leupeptin and aprotinin was added in a final concentration of 1 μg/ml. Protein concentration was measured by the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL, USA). Primary antibodies against the proteins and appropriate conjugates were obtained from DAKO (Glostrup, Denmark), recombinant Myeloid Related Proteins (MRP) 8/14, as well as polyclonal rabbit antibodies against them, were a kind gift from Dr. Claus Kerkhoff (Institute of Experimental Dermatology, Muenster, Germany). In addition, we produced affinity chromatography purified monoclonal antibodies against human heat shock protein 60 (HSP60) from clone II-13 (Xu et al., 1994).

2.3. Immunodetection in situ (silicone-linked immunosorbent assay, SILISA)

Polystyrene flat-bottom 96-well microtiter plates (Model 655180, Greiner, Frickenhausen, Germany) were coated with 50 μl/well of various silicone types and immediately centrifuged (10 min at 1000*g*) to distribute the material evenly on the bottom of the wells. Since silicone type MED-1511 (Nusil; Waldbroon, Germany) is one of the most frequently used for silicone implants, this material was tested as a paradigmatic example. However, silicone types MED-4860, -4211 and -6604 have also been found to be compatible with this detection system. After silicone curing, plates were sterilized for 200 s under UV (200 mJ, GsGene UV chamber, Biorad, Hercules, USA), and incubated with 30 μl/well of sterile serum supplemented with protease inhibitors for 50–100 hours. Plates were then washed twice in phosphate buffered saline (PBS, pH 7.2) and distilled H_2O at 4 °C. These plates were immediately used for protein analysis, either by protein elution or by SILISA. The process is depicted schematically in Fig. 1.

Table 1
Antibodies used for the SLISA to detect protein adhesion to silicone

Antibody against	Dilution	Company, code no.
Mouse isotype control	1:1000	DAKO X931
Rabbit isotype control	1:1000	DAKO X903
Fibronectin	1:1000	DAKO A245
CRP	1:500	DAKO A073
Collagen III	1:500	Chemicon AB 747
MRP 14	1:500	C. Kerkhoff[a]
Collagen I	1:500	Calbiochem 234167
C3 complement	1:500	DAKO M0836
IgG-HRP	1:500	DAKO P 214
HSP 60	1:1000	Clone II-13[a]

Seven candidates distinguishing fibrotic patients emerged from the proteomics screen of 184 proteins that adhere to silicone. These candidates show differences in protein adhesion between patients with and without fibrotic responses to silicone. Antibodies against the seven proteins, their isotype controls (DAKO X903, DAKO X910), respective dilutions used and producers are given in the table.

[a] Antibodies produced in our facilities or obtained through collaborative projects.

Binding sites on the plates were blocked with 2.5% BSA/0,1% Tween 20 in PBS for 90 min on an orbital shaker at room temperature. Subsequently, various antibodies were applied and diluted in blocking solution (2.5% BSA/0,1% Tween 20 in PBS). Our initial screening pool was composed of 30 antibodies, and differences between patients with fibrosis vs. controls ($p<0.05$) were observed with 7. The antibodies, suppliers and respective dilutions of antibodies are given in Table 1. After incubation with the primary antibodies, the plates were washed 3×5 min with PBS, incubated 120 min with appropriate secondary antibodies conjugated with HRP [rabbit anti-mouse (DAKO P0260) or swine anti-rabbit (DAKO P0399) IgG], diluted in blocking solution on a shaking platform at room temperature. For some proteins, antibodies directly labeled with HRP were used. After incubation with these antibodies, the wells were incubated with PBS until the last step. After the conjugate incubation, the plate was washed 3×5 min in PBS/0.02% Tween, and 2×5 min in PBS. A standard acid solution of 2, 2′-azino-bis (3-ethylbenzthiazoline-6) sulphonic acid was used to monitor the activity of HRP in conjugates. The enzymatic color reaction was developed for 40 min and absorption measured at 405 nm (Tecan, Maennedorf, Switzerland). The cutoff for presence of a protein was defined as 2 absorbance values of isotype controls. The final results were calculated after subtraction of isotype control values. Since absorbencies rather than absolute protein concentrations are being measured, we incubated plates coated with the same silicone type with standard protein solutions of purified human fibronectin, MRP14, IgG and HSP60, and detected their adhesion using the same immunodetection *in situ*-test system.

Not all proteins afforded absorbance values with normal distribution, and they were, therefore, log-transformed using the formula $x'=\log(x+1)$. Newly transformed data sets showed a normal distribution, as confirmed by the Kolmogorov–Smirnov test, and were used for further statistical analysis (Ryder and Robakiewicz, 2004). After transformation, a factor analysis was performed to determine the minimal number of parameters needed for successful discrimination of patients with

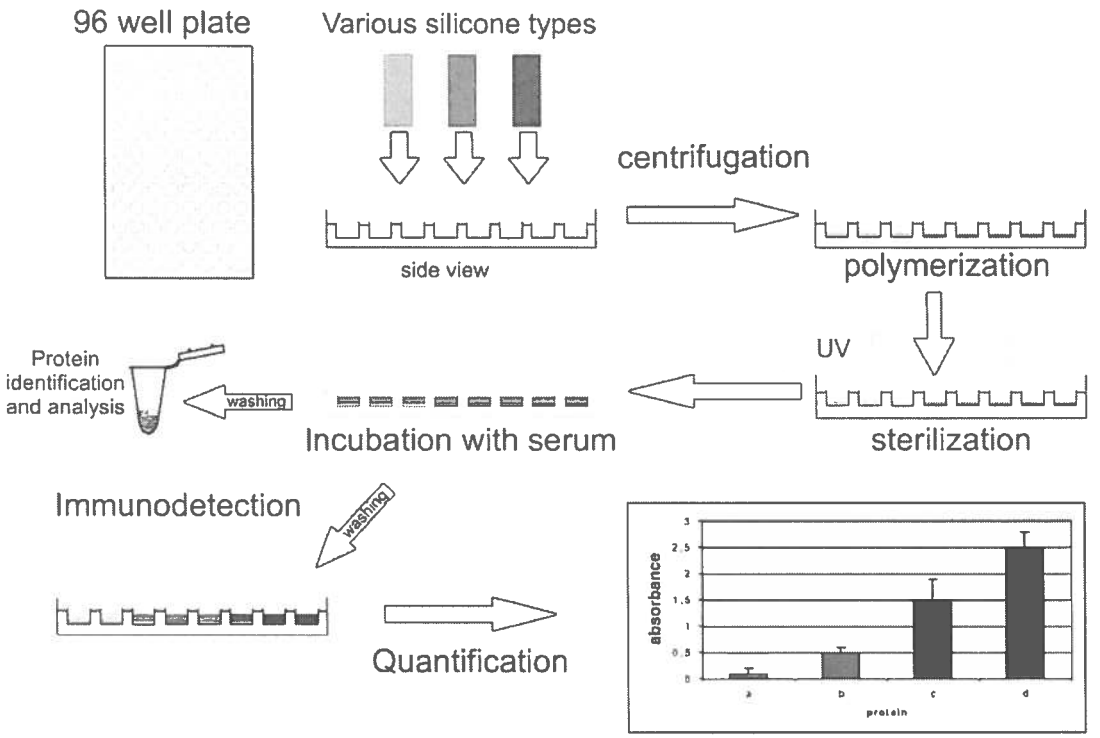


Fig. 1. Silicone-linked immuno-sorbent assay (SILISA). Various medical grade silicone types were used to coat the bottom of microtiter plates and centrifuged to distribute the silicone evenly on the bottom of the wells. After silicone curing, the plates were sterilized under UV and incubated with serum for 50 hours. Unbound proteins were then washed away, and adherent proteins either eluted for biochemical characterization or detected *in situ* with appropriate antibodies and HRP-labeled conjugates. Absorbencies were subsequently measured at 405 nm in a conventional ELISA reader and obtained measured values statistically processed using SPSS software.

fibrotic side effects from non-affected controls. Having established a minimal number of discriminatory parameters, logistic regression was used to formulate a model system that could quantify the differences between the groups. Statistical evaluation, factor analysis and logistic regression were performed using SPSS statistical package software (SPSS Inc. Chicago, Illinois).

3. Results

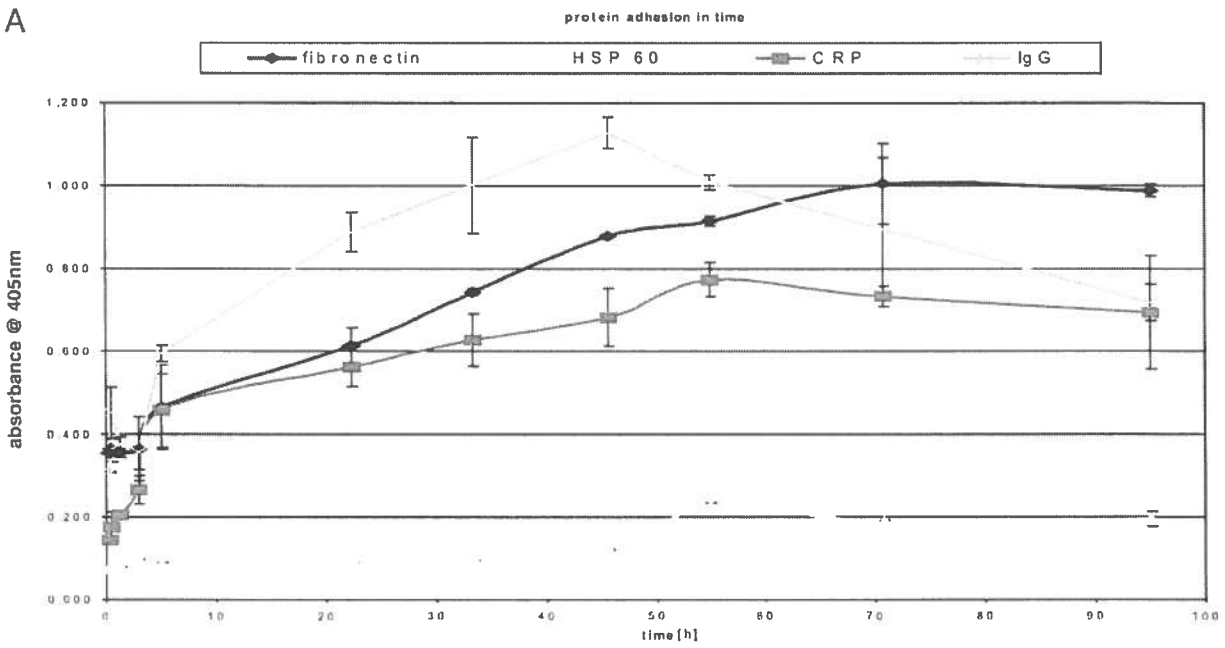
3.1. Protein adhesion kinetics

Using the SILISA, deposition of four proteins important for the host response to silicone was monitored in time. Antibodies against fibronectin, IgG, CRP and HSP 60 were used to assess deposition of these proteins to silicone. These proteins were chosen from the total set of proteins deposited on the silicone because their role in host defence to foreign materials is well-documented, they are known to play a prominent role in subsequent fibrotic processes, and their molecular weights (from 200–14 kD) allow probing a wide range of serum proteome. Results are presented in Fig. 2A as time-scaled averages ± standard deviation from triplicates in a representative experiment. The figure shows the dynamic processes of proteinaceous film formation on the surface of silicone.

To determine the correlation of *in situ* absorbance to the absolute protein amount deposited, standard protein solutions ranging from 1 μg to 10 ng were applied per cm^2 of silicone-coated 96-well plates. After 50 hours, the plates were washed with PBS and protein adherence assessed by the SILISA system. Linear dependence was detected in the entire range tested ($R^2 > 0.95$), allowing for simple recalculation of protein amounts from measured absorbencies. However, it should be emphasized that proteins adhere differently when presented in complex protein mixtures compared to single protein solutions, which is why these findings are considered an orientation rather than an absolute measure of protein deposition to silicone surfaces.

3.2. Regression analysis

Initial fibronectin concentration in 20 randomly selected sera was measured at the Central Laboratory



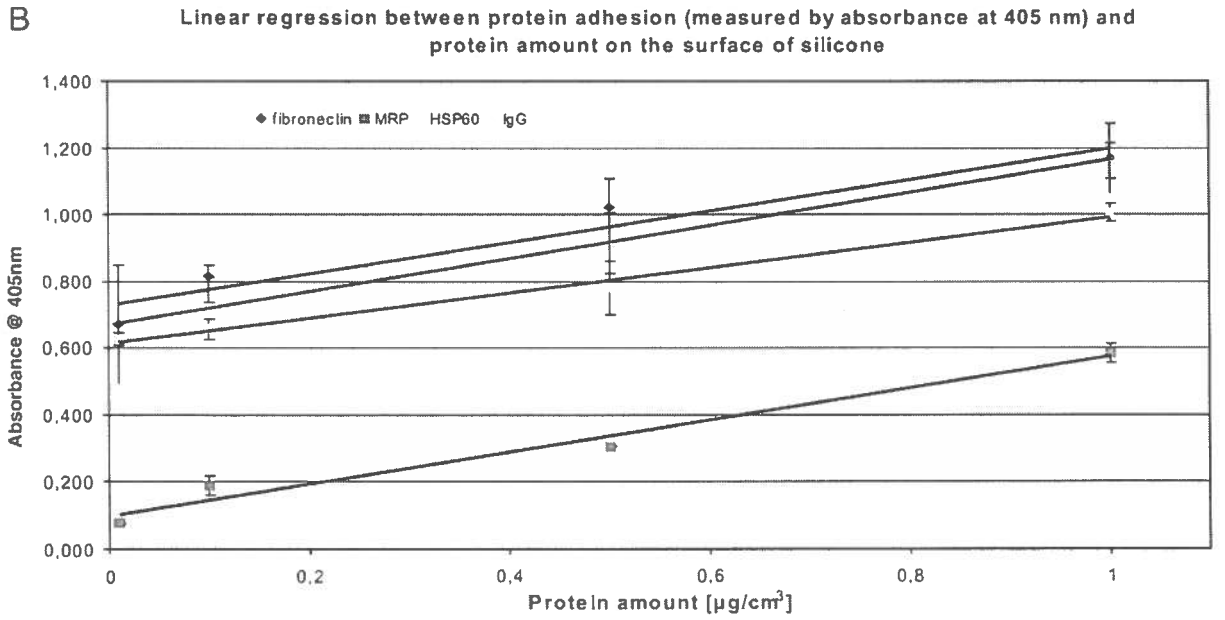


Fig. 2. A. Time scale of protein adhesion to silicone. Serum was added to silicone type MED-1511-coated microtiter wells and excess unbound proteins were washed away at various time points. Adhering fibronectin, C-reactive protein (CRP), immunoglobulin G and heat shock protein (HSP) 60 were tested in the SILISA. Average values of optical densities at 405 nm±SD are shown for each time point. While the amount of HSP60 remained constant throughout the assay, maximal CRP deposition was reached after 40 hours, and IgG and fibronectin after 50 hours. Note rapid degradation/protein displacement of IgG that begins as early as 60 hours. B. Correlation between protein adhesion, measured as absorbance at 405 nm, and absolute protein amount on the surface of silicone. Standard protein solutions of fibronectin, MRP, HSP60 and IgG were incubated in concentration from 1 µg/cm^2 to 10 ng/cm^2 for 50 hours. Unbound proteins were washed away with PBS, and the remaining ones were detected using SILISA for protein adhesion. Regression between protein adhesion and absolute protein amount was linear (R^2>0.95) in this range with the following functions: y=0.4689x+0.7303 for fibronectin; y=0.4947x+0.6695 for IgG; y=0.3786x+0.6155 for HSP 60 and y=0.4769x+0.0976 for MRP14. Although adhesion of those proteins show straightforward linear dependence with their concentration, this correspondence is present only in pure protein solutions, and is not evident in complex mixtures such as serum.

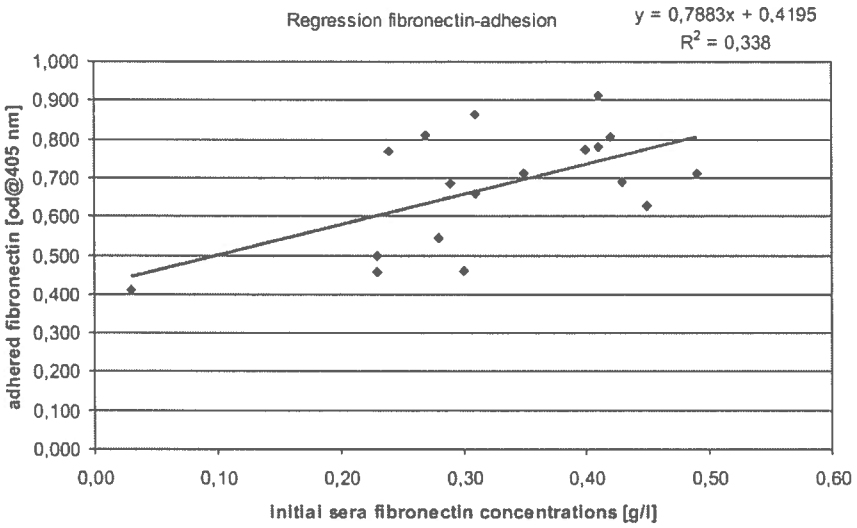


Fig. 3. Regression analysis for fibronectin deposition and concentration of fibronectin in serum. Fibronectin concentration was measured in 20 randomly selected sera that were simultaneously incubated with silicone. After 50 hours, the relative adhesion of fibronectin, as determined by SILISA assay, was measured and plotted against the initial fibronectin serum concentrations. Contrary to the pure protein solutions, where protein concentration and adherence to silicone surface show linear dependence, this phenomenon is not evident in complex protein mixtures such as serum. Correlation of serum fibronectin concentration and adhesion to silicone, as measured by Pearson correlation coefficient (R^2), showed no direct correlation between these two variables.

Unit of the Innsbruck University Clinic. Identical sera were simultaneously incubated with silicone, and deposited fibronectin was detected using the SILISA assay, as described earlier. The two variables were plotted on the same graph, and correlation was estimated using the model of linear regression and calculating Pearson's coefficient of linear regression, R^2, (Fig. 3). The same correlation analysis was performed for IgA (R^2=0,741), IgG (R^2=0,6042), C-reactive protein (R^2=0,074), HSP60 (R^2=0,0547) and MRP8/14 (R^2=0,1756) (Pearson's coefficient values are given in the brackets for respective proteins). The poor correlation of initial serum protein concentration and absorbance values is a consequence of "matrix effect", which describes different adhesion of proteins from complex mixtures rather than from pure/simple protein solutions (Lacy et al., 2002).

3.3. High throughput detection of protein deposition to silicone

Women undergoing cosmetic breast augmentation are a unique population of otherwise healthy individuals that can be considered paradigmatic in that they develop various degrees of adverse reactions to silicone without confounding variables. In a previous study (Backovic et al., 2007), we identified proteins that are deposited on the surface of silicone. Of 184 proteins detected, the 30 most prominent were selected in our initial SILISA system screen, and our factor analysis and cross-correlation tables further reduced these to 7 proteins that correlated significantly with fibrosis. Deposition patterns of those 7 proteins were subsequently compared in a cohort study (Fig. 4), as described in Methods section. In our experiments, background values were less than 1% of probe measurements. During analyses, we noticed a group of controls (20%, N=8) with a protein deposition profile analogous to that of fibrosis patients, and this group was classified as a separate group of "fibrosis-prone controls". After we separated the cohort into three groups (controls without fibrosis, patients with fibrosis, and "fibrosis-prone" controls), the differences in protein deposition patterns between the groups was quantified using principal component analysis (PCA), with controls without fibrosis as the reference to which the other groups were compared (Fenollar et al., 2006). The model constructed upon PCA was used to reprobe the same cohort, and it correctly identified 87.5% of controls without fibrosis, 92.9% of patients with fibrosis and 80% of "fibrosis-prone" controls. The details of the PCA classification are given in Table 2.

This finding was further confirmed by clustering the data using "Treeview" clustering software (Eisen et al., 1998), the results of which are shown in the Fig. 5. Patients with fibrosis were clustered in a group separate

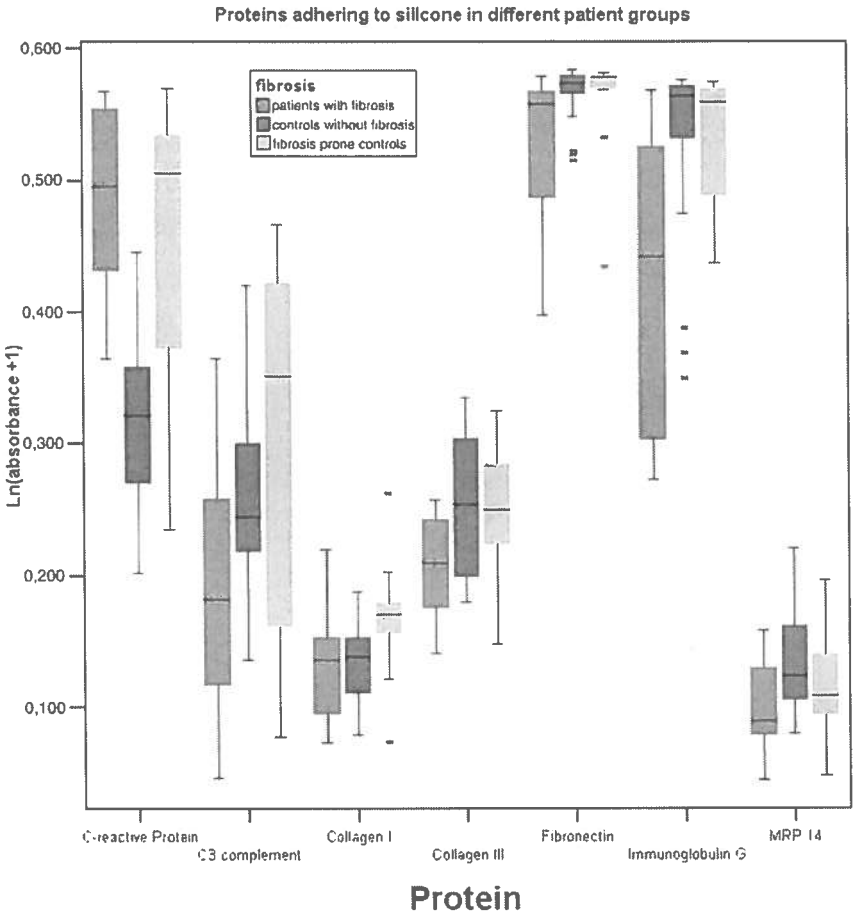


Fig. 4. Protein adhesion to silicone in a cohort of women with silicone mammary implants (SMIs). Diagram shows box-plots with extremes (–) of ln-transformed absorbance measurements of proteins adhering to silicone in 3 different patient groups. Adhesion of seven selected proteins was simultaneously detected in 70 women with SMIs. Those with strong fibrotic reactions to silicone (patients with fibrosis; Baker score ≥ 3) showed different protein deposition patterns compared to those without adverse reactions (controls without fibrosis; Baker score < 3). In the course of factor analysis that was used to quantify the differences between groups, a fraction of individuals among controls without fibrosis showing "fibrotic" protein deposition patterns emerged, and they are depicted as separate group of "fibrosis-prone controls". Details on nominal regression and statistic analysis of the differences between groups are given in the text.

from patients without. Analogous to the graph in Fig. 4, 20% of control patients were clustered in the same group as patients developing a fibrotic reaction to silicone.

4. Discussion

Silicone biocompatibility is restricted by the effects of proteins adhering to their surfaces on the surrounding

Table 2
Classification of fibrotic cases after the application logistic regression model

	Predicted			
Observed	Controls without fibrosis	Patients with fibrosis	"Fibrosis-prone" controls	Percent correct
Controls without fibrosis	14	1	1	87.5
Patients with fibrosis	1	26	1	92.9
"Fibrosis-prone" controls	1	4	20	80.0
Total percent	23.2	44.9	31.9	87.0

Thirty proteins were tested in a SILISA assay and only the seven of those whose absorbance values correlated with fibrosis status were used for further analysis. The most correct model was obtained after introducing the group of "fibrosis-prone" controls into logistic regression algorithm. Re-probing the same sample, this time blinded for the fibrosis outcome, the model classified correctly 87% individuals into the three named groups.

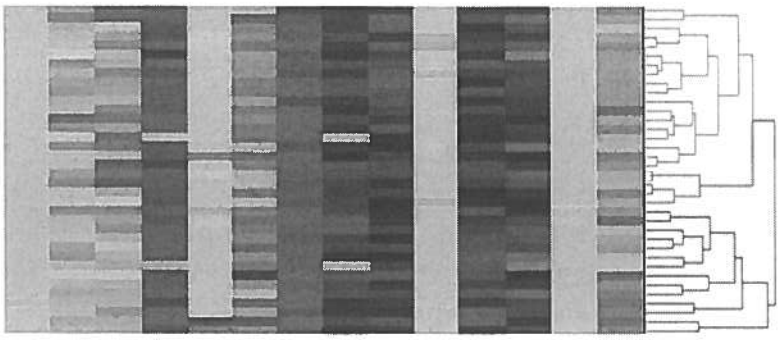


Fig. 5. Cluster analysis of the protein adhesion data from the cohort of women with SMIs. Cluster analysis was used to confirm the results obtained from the nominal regression. The same group of "fibrosis-prone controls" was clustered together with patients with fibrosis using "Treeview" clustering software. Patients with fibrosis were clustered (red branch) in a separate group from control patients that did not develop fibrosis (black branch). "Fibrosis-prone controls" (green branch) were clustered together with patients who developed a fibrotic reaction to silicone.

tissue (Tang and Eaton, 1999; Shen et al., 2004). Cells involved in a foreign body response to biomaterials interact with implanted devices indirectly, recognizing proteins adhered on their surface. However, the multidimensional network of stimuli provided by adhered proteins to various neighboring cells cannot be elucidated without recording the dynamic adherence processes of the proteome (Sachs et al., 2005; Canelle et al., 2005). Our approach combined results from a wide proteomics-based screening to construct a new test system for detection of protein adhesion patterns.

Insight into the early stages of proteinaceous film formation provides a list of potential modulators of the local and systemic immune responses to silicone. We have previously identified numerous serum proteins adhering to the surface of silicone on a proteome-wide scale (Backovic et al., 2007; Wick et al., 1987) that, as tentative initiators and/or modulators of the response to silicone, were considered to be potential prognostic and therapeutic candidates. Our current work describes the kinetics of their adhesion and demonstrates that protein adhesion to silicone is largely independent of relative molar ratios of given proteins in the surrounding tissue, fibronectin being an important example (Fig. 3).

Up on the initial proteomics screen of the proteins deposited to silicone, we tested numerous protein candidates with the SILISA system. Based on the results of factor analysis, we focused on 7 proteins that showed different silicone adhesion properties in patients with fibrosis compared to patients without strong fibrotic reactions to silicone. We discuss two that may have an important role in the development of fibrotic reactions to silicone biomaterials.

In view of the fact that expression of HSP 60 has been demonstrated at the site of various forms of stress, including mechanical stress, this protein might play an important functional role in the development of the proteinaceous film on silicone implants. We believe that HSP60 detected on the surface of silicone reflects the response of tissue surrounding SMIs to mechanical stress caused by SMI movement under physiological conditions. The same pleiotropic antagonism of HSP 60 has been described in several diseases that entail autoimmune responses, such as rheumatoid arthritis and atherosclerosis (Van Eden, 2003; Wick et al., 2004). Furthermore, we previously found strong HSP60 expression in the multilayered "pseudosynovia" consisting of fibroblasts and macrophages abutting the silicone mammary implants (Wolfram et al., 2004). In this context, HSP 60 most likely mediates dendritic cell-mediated Th1 response, which favors the development of local fibrosis and various autoimmune phenomena (Flohe et al., 2003).

To our knowledge, this is the first report of adhesion kinetics of myeloid-related protein 14 (MRP14, calprotectin) on the surface of silicone implants. This protein (in a complex with MRP8) plays an important role in leukocyte recruitment, enhances adhesion to circulating monocytes (Bouma et al., 2004; Berntzen et al., 1991) and is found in increased extracellular concentrations at local sites of inflammation (Nacken et al., 2003; Fietta et al., 2006). It is, therefore, an interesting candidate within the concept of immune system-mediated adverse reactions to silicone. Since MRP8/14 induces transcription of proinflammatory chemokines and adhesion molecules in microvascular endothelial cells (Viemann et al., 2005), stimulates fibroblast growth, and inhibits matrix metalloproteinsases (Shibata et al., 2004; Isaksen and Fagerhol, 2001), it might exert those effects locally on the surface of silicone, initiating the adverse reaction. This is supported by the observed individual differences in MRP8/14 deposition on the silicone surfaces that correlate with the development of adverse reactions.

Our main task was to clarify whether differences in individual adhesion properties of the protein array are responsible for the variations observed in development of subsequent adverse reactions to silicone devices. Our system allowed for simultaneous analysis of deposition of numerous proteins in a cohort of patients, and successfully distinguished patients in which fibrosis was developing in comparison to those without such complications. We initially focused only on the few proteins that showed differences in adhesion properties in individuals with strong adverse effects to implanted biomaterial. However, in patients without detectable fibrotic processes, a subgroup with a deposition pattern analogous to the patients who were developing fibrosis in response to silicone was identified. Interestingly, the percentage of these patients corresponds to the incidence of complications as a result of silicone implants (VandeVord et al., 2004; Brandon et al., 2003; Kossovsky and Stassi, 1994). A follow-up study is now being conducted to determine if this group is at potential risk of strong adverse fibrotic reaction to silicone as well as a to fibrotic processes in general, such as hypertrophic scar formation, infection, alcoholic liver damage, rheumatoid disease etc.

Fibrotic processes in the peri-implant capsule are largely mediated by the mechanisms of the host response that are initiated through the interaction with the proteins on the film formed on the implant surface. The protein adsorption test system shown here can be used to test the adhesion properties of proteins on a genome-wide scale, and the protein signature on the surface of various medical silicone types can also be probed to test their biocompatibility. Whether there is a causative relation between autoimmune syndromes and active and passive silicone implants is still debatable. However, our findings contribute to the understanding of this inflammatory network, and might prove useful in the modification of implant design and targeted therapy of patients with fibrosis.

Acknowledgments

This project has been supported by the Kompetenzzentrum Medizin Tirol (KMT), and a grant from the Lore and Udo Saldow Foundation. The authors thank Dr. C. Kerkhoff for the antibodies provided, C. Mayerl for technical support and M. K. Occhipinti-Bender for editorial assistance. The serum fibronectin concentrations were measured in the Central Laboratory of Innsbruck University Clinic (head A. Griesmacher). Work in the Huber laboratory is supported by the Austrian Proteomics Platform (APP) within the Austrian Genome Program (GEN-AU), Vienna, Austria.

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