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Coating with genetic engineered hydrophobin promotes growth of fibroblasts on a hydrophobic solid

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Abstract

Class I Hydrophobins self-assemble at hydrophilic–hydrophobic interfaces into a highly insoluble amphipathic film. Upon self-assembly of these fungal proteins hydrophobic solids turn hydrophilic, while hydrophilic materials can be made hydrophobic. Hydrophobins thus change the nature of a surface. This property makes them interesting candidates to improve physio- and physico-chemical properties of implant surfaces. We here show that growth of fibroblasts on Teflon can be improved by coating the solid with genetically engineered SC3 hydrophobin. Either deleting a stretch of 25 amino acids at the N-terminus of the mature hydrophobin (TrSC3) or fusing the RGD peptide to this end (RGD-SC3) improved growth of fibroblasts on the solid surface. In addition, we have shown that assembled SC3 and TrSC3 are not toxic when added to the medium of a cell culture of fibroblasts in amounts up to 125 µg ml⁻¹.

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1. Introduction

Artificial materials for implant surfaces can be used to replace or support a variety of body parts including bone, spinal, cardiac and dental tissues. The non-physiological character of these materials often leads to poor integration into human tissue and makes it necessary to develop implant materials that have improved biocompatibility. By modifying the physio- and physico-chemical surface properties of the biomaterial its interaction with cells can be improved. For instance, mouse fibroblasts attach better to moderately hydrophilic surfaces than to hydrophobic surfaces [1]. Similarly, human endothelial cells and foreskin

fibroblasts adhere optimally on moderately wettable polymers [2–4]. Another approach to improve biocompatibility is to modify the material surface with biomolecules involved in receptor-mediated cellular functions [5], such as fibronectin. Fibronectin is a protein present in extracellular matrices and is involved in cell adhesion. The Arg–Gly–Asp (RGD) sequence of this adhesin binds to integrins located in the cell plasma membrane [6–8]. By immobilizing this tripeptide at an implant surface, cell attachment is promoted in a way similar to that of fibronectin [9–15]. The RGD peptide is preferred over fibronectin in tissue engineering since it can be synthesized chemically in large quantities, it is not prone to denaturation and can be easily fused to proteins by genetic engineering.

Class I hydrophobins seem very attractive to change the physio-chemical and physical–chemical properties of surfaces of implant materials and/or to immobilize peptides like RGD at surfaces. These secreted proteins

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fulfil a broad spectrum of functions in fungal growth and development [16–18]. For instance, they coat fungal aerial structures such as spores (e.g. those present in blue cheeses) and fruiting bodies (including edible mushrooms), thus making them water repellent. Hydrophobins are about 100 amino acids in length and characterized by eight conserved cysteine residues and a conserved spacing of hydrophilic and hydrophobic regions [16]. However, their amino acid sequences are diverse. The most characteristic feature of class I hydrophobins is that they self-assemble at any hydrophilic/hydrophobic interface (e.g. between water and air, water and oil, or water and a hydrophobic solid-like Teflon) into a 10 nm thin highly insoluble amphipathic film [19–21]. The insolubility of the film discriminates class I hydrophobins from the class II hydrophobins. The latter hydrophobins also self-assemble but their films readily dissociate in for instance 60% ethanol or diluted detergent [22,23] and also by applying pressure or by cooling [22]. The wettability of the hydrophilic side of assembled class I hydrophobins is highly variable and depends on the nature of the hydrophobin. In contrast, the wettability of the hydrophobic side of the film is extremely low in all cases (similar to that of Teflon) [24]. By self-assembly, hydrophobins change the wettability of a surface. Glass and filter paper turn hydrophobic by drying down a solution of hydrophobin on these surfaces [19,25–27]. Conversely, hydrophobic solids (e.g. Teflon or polystyrene) or liquids (e.g. paraffin oil) can be made hydrophilic by submerging or suspending these materials into a solution of hydrophobin [20,21,25–27]. Assembled class I hydrophobins (e.g. SC3 of *Schizophyllum commune*) strongly interact with hydrophobic solids. For instance, they resist washes with water or diluted detergent at 100°C [20,21].

Genetically engineered derivatives of the SC3 hydrophobin of *S. commune* were obtained by deleting 25 amino acids at the N-terminal part of mature SC3 (TrSC3) and with the RGD tripeptide fused at the N-terminus of the mature forms of SC3 and TrSC3 (RGD-SC3, RGD-TrSC3) [28]. These modifications did not affect the gross properties of the hydrophobin. However, they changed the biophysical properties of the hydrophilic side of the assembled protein. TrSC3 and RGD-TrSC3 had a decreased wettability at this side compared to that of SC3 and RGD-SC3. In a qualitative study it was shown that growth of fibroblasts on Teflon coated with TrSC3, RGD-SC3 or RGD-TrSC3 was improved compared to that on bare Teflon or SC3-coated Teflon [28]. We here assessed whether assembled SC3 and TrSC3 are toxic when added to the medium of fibroblasts and studied quantitatively the growth of fibroblasts on Teflon coated with the genetic engineered hydrophobins.

2. Materials and methods

2.1. Growth conditions for *schizophyllum commune*, isolation and purification of hydrophobins

S. commune was grown in 11 shaken cultures (225 rpm) for 5–7 days at 24°C in production medium [28]. For production of the SC3 hydrophobin, a strain was used in which the *SC15* gene was deleted [29]. RGD-SC3, TrSC3 and RGD-TrSC3 were purified from the medium of *S. commune* strains in which the *SC3* gene was deleted [20,30], and in which constructs were introduced with coding sequences of the SC3 derivatives placed under control of the SC3 regulatory sequences [28]. SC3 and RGD-SC3 were purified as described by Wösten et al. [19] and Wessels [16], while purification of TrSC3 and RGD-TrSC3 was done according to Scholtmeijer et al. [28]. SC4 was produced using a dikaryon of *S. commune* resulting from a cross between strain M4.58 [31] and strain 72-3 [20,30]. The former strain has its *SC3* gene deleted and expresses the *SC4* gene under control of the SC3 regulatory sequences. In the latter strain only the *SC3* gene is deleted. SC4 was purified like SC3.

2.2. Cell culture

Mouse fibroblast strain L929 (ATTC CCL-1) was cultured in 25 cm² T-flasks in RPMI-1640 medium supplemented with penicillin and streptomycin (1% each), 1% L-glutamine and 10% fetal calf serum. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ and routinely passaged by trypsinization. All products were purchased at Life Technologies.

2.3. Cytotoxicity of monomeric and assembled hydrophobin

To test cytotoxicity of monomeric hydrophobin, the protein was monomerized with trifluoroacetic acid (TFA) as described [19] and solubilized in RPMI medium (250 µg ml⁻¹). This stock solution was diluted into the supplemented RPMI medium (see above) to the desired concentration of hydrophobin and used to replace the medium of cultures that had grown for 24 h in test wells starting from a density of 7500 cells cm⁻². Growth was prolonged for 72 h. To test cytotoxicity of hydrophobin assembled at the water/air interface, the same procedure was used but the stock solution of monomeric hydrophobin in RPMI was vortexed 15 min to allow the protein to self-assemble. Cells were grown in multi-well tissue culture polystyrene (TCPS) plates (6-/12-/24-well) to evaluate cell morphology and to quantify cell growth, while 24 or 96

multi-well plates were used to determine cell activity (MTT test, see below).

2.4. Cytocompatibility of hydrophobin-coated Teflon

Teflon sheets (FEP; 0.25 mm thick; purchased from Norton Fluorplast, Raamsdonkveer, The Netherlands) were cleaned with 70% ethanol and sterilized at 123°C. These sheets were incubated overnight in a sterile aqueous hydrophobin solution ($50 \mu\text{g ml}^{-1}$), thus allowing the protein to self-assemble at the surface of the Teflon. After washing with sterile water, the sheets were allowed to dry. Reproducibility of the coating of the sheets was confirmed by water contact angle measurement [32]. Bare or coated Teflon sheets were cut into discs of 1.75 cm^2 to cover the bottom of 24 well plates. Cells were seeded into the test wells to a density of $7500 \text{ cells cm}^{-2}$. Cell morphology, cell numbers and cell activity were monitored at 24 h intervals for 96 h.

2.5. Analysis of cell cultures

Confluence of cultures and morphology and lyses of cells were monitored using light microscopy. To quantify cell numbers, cells were harvested with a 0.25% trypsin/0.3% EDTA solution in PBS and counted using a haemocytometer. Mitochondrial activity of cells was evaluated by measuring the conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazo-

lium bromide) into its formazan product, which can be quantified at 590 nm using an ELISA reader [33].

2.6. Statistical analysis

All experiments were performed at least in triplicate. Results are expressed in mean $\pm$ standard deviation of the mean. Comparative analyses were performed with SPSS 10.0 for Windows using paired samples *T*-test at 95% confidence level. Unless stated otherwise *p* was smaller than 0.05.

3. Results

3.1. Cytotoxicity of monomeric and assembled SC3 and TrSC3

To investigate whether the SC3 and TrSC3 hydrophobins have cytotoxic effects, monomeric and assembled forms of the hydrophobins (1.25 – $125 \mu\text{g ml}^{-1}$) were added to fibroblast cultures that had grown for 24 h to a confluence of 15–20% and growth was prolonged for 72 h. Confluence, cell morphology and cell lysis of cultures either or not containing hydrophobin were similar throughout this period. After 24 h confluence of the cultures was 25–30%, while it increased to 50–60% and 85–90% after 48 and 72 h, respectively (not shown). Confluence correlated with the cell numbers (Fig. 1). A lower cell number was only

Fig. 1. Cell numbers after growing fibroblasts 24 h (a, b) or 72 h (c, d) in the presence (1.25 – $125 \mu\text{g ml}^{-1}$) or absence of monomeric or assembled forms of SC3 (a, c) or TrSC3 (b, d). Open bars represent monomeric hydrophobin, gray shaded bars the assembled forms, while the hatched bars represent cultures grown in the absence of hydrophobin. Error bars indicate standard deviation of the mean.

observed after 24 h of exposure to 125 µg ml⁻¹ monomeric TrSC3.

Mitochondrial activity of the cells decreased in the presence of hydrophobin. In case of the SC3 hydrophobin, mitochondrial activity was only similar to the control well when cells were grown for 24 h and exposed to 1.25 µg ml⁻¹ monomeric protein (*p* = 0.6) (Fig. 2). In all other cases mitochondrial activity was 50–80% of that in the control well after 24 h of growth (Fig. 2), while it was 40–60% after 48 h (not shown) and 70–80% after 72 h of growth (Fig. 2). No differences were observed between monomeric and assembled SC3. In case of TrSC3, mitochondrial activity was reduced maximally 35% after 24 h (Fig. 2), while after 48 h (data not shown) and 72 h of growth (Fig. 2) a reduction of maximally 45% was observed. Assembled TrSC3 reduced mitochondrial activity stronger than the monomeric form.

3.2. Cytocompatibility of Teflon after assembly of SC3 and its derivatives

Teflon sheets were incubated overnight in aqueous solutions of the SC3 hydrophobin or one of its derivatives. After washing with water and drying, wettability of the sheets was determined by measuring the water contact angle (Table 1). SC3 and RGD-SC3 made the surface highly wettable, while Teflon coated with TrSC3 and RGD-TrSC3 was moderately hydrophilic.

To investigate whether cytocompatibility of Teflon was improved after coating with SC3 or one of its derivatives fibroblasts were grown for 96 h on bare or coated Teflon discs that covered the bottom of a well. Cells grown directly on TCPS served as a control. Confluence, cell morphology, cell numbers and mitochondrial activity were monitored. Cell numbers were highly variable after 24 h (Fig. 3) and 48 h of growth (not shown). However, after 72 h (not shown) and 96 h (Fig. 3) variation leveled down. After 96 h of growth, the cell number was lower on SC3-coated Teflon than on bare Teflon, amounting 40% and 50%, respectively, relative to that on TCPS. In both cases cells were rounded indicating poor attachment (Fig. 4). Compared to SC3-coated Teflon, cell numbers were higher on Teflon sheets coated with RGD-SC3, TrSC3 or RGD-TrSC3. Cell numbers found on Teflon coated with derivatives of the SC3 hydrophobin were 60–70% of those in the control well (Fig. 3). These findings

Table 1
Water contact angles of Teflon after coating with hydrophobins

Hydrophobin used for coating Teflon	Water contact angles
Bare Teflon	115 ± 2
SC3	36 ± 1
RGD-SC3	46 ± 8
TrSC3	69 ± 2
RGD-TrSC3	57 ± 4
SC4	60 ± 2

Fig. 2. Mitochondrial activity as assessed by the MTT test of cells grown for 24 h (a, b) or 72 h (c, d) in the presence (1.25–125 µg ml⁻¹) or absence of monomeric or assembled forms of SC3 (a, c) or TrSC3 (b, d). Open bars represent monomeric hydrophobin, gray shaded bars the assembled forms, while the hatched bars represent cultures grown in the absence of hydrophobin. Error bars indicate standard deviation of the mean.

Fig. 3. Cell numbers (a, b) and mitochondrial activity as assessed by the MTT test (c, d) of cultures grown on bare Teflon or Teflon coated with SC3 or one of its derivatives for 24 h (a, c) or 96 h (b, d). Error bars indicate standard deviation of the mean.

Fig. 4. Fibroblasts cultures grown for 96 h at surfaces of bare Teflon and Teflon coated with SC3, RGD-SC3, TrSC3 and RGD-TrSC3, TCPS serving as a control. Bar indicates 100 μm.

correlated with the estimated confluence of the wells (Fig. 4). Confluence of the cultures grown in the control well and those grown on TrSC3-coated and RGD-TrSC3-coated Teflon was 95%. In contrast, it was 80% in case of bare Teflon and RGD-SC3-coated-Teflon and 70% for SC3-coated Teflon. Morphology of the cells grown on RGD-TrSC3 and TrSC3 was similar to that in the control well, while the morphology of cells grown on RGD-SC3-coated Teflon was intermediate between bare Teflon and the control well. Throughout culturing, mitochondrial activity of cultures grown on bare Teflon was 30% of that in the control well (Fig. 3c and d), while the activity of cells cultured on hydrophobin-coated Teflon was reduced to 25–60%. These reduced activities can be explained to a large extent by the reduced cell numbers (see Fig. 3a and b).

To obtain an indication whether the degree in wettability or the differences in chemical composition favors cell growth in case of TrSC3, growth was compared to that on SC4-coated Teflon. The assembled SC4 hydrophobin exposes a hydrophilic side with a wettability similar to that of TrSC3 (Table 1) but shares an identity in amino acid sequence of only 45% (Fig. 5). Cell morphology, cell lysis and confluency were similar (data not shown) as well as cell numbers (Fig. 3). However, mitochondrial activity was lower in case of SC4.

4. Discussion

Class I Hydrophobins change the nature of a surface by their ability to self-assemble at hydrophilic-hydrophobic

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SC3  : GGHPGTTTPPVTTTVTTPPSTTTIAAGGTCTTGSLSCCNQVQSASSSPVTALLG-LLG
TrSC3: GGHPGTCTTGSLSCCNQVQSASSSPVTALLG-LLG
SC4  : GGGKGAGQACNSGPVQCCNETTTVANAQKQGLLGLLG

SC3  : IVLSDLNVLVGISCSPLTVGVG-GSGCSAQTVCCEN-TQFNGLINIGCTPINIL
TrSC3: IVLSDLNVLVGISCSPLTVGVG-GSGCSAQTVCCEN-TQFNGLINIGCTPINIL
SC4  : VVVGPITGLVGLNCSPISVVGVLTGNSCTAQTVCCDHVTQ-NGLVNVGCTPIS-L

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Fig. 5. Alignment of SC3, TrSC3 and SC4 of *S. commune*. Amino acids in bold are conserved. The eight conserved cysteine residues were used as fixed coordinates.

interfaces into a 10 nm highly insoluble amphipathic film. Hydrophilic surfaces can be made hydrophobic, while hydrophobic surfaces can be made hydrophilic. The amphipathic film strongly interacts with hydrophobic supports like Teflon resisting washes with water or hot diluted detergents [20,21,24]. Hydrophobins present a generic way to change the nature of hydrophobic surfaces because the interaction with the solid is non-covalent and is thus not dependent on available groups for cross-linking. Although the hydrophobic side of assembled class I hydrophobin is highly water repellent in all cases, the degree of wettability at the hydrophilic side depends on the hydrophobin being used. These properties make hydrophobins interesting candidates for use in various applications including implants and medical devices [16,28].

In case of medical applications, a hydrophobin should not be immunogenic or toxic, while it should promote growth of particular cells when used for implants. Low antibody titers, if any, were obtained when class I hydrophobins (e.g. SC3 and SC4 of *S. commune*) were injected subcutaneous into rabbits, indicating that hydrophobins are hardly immunogenic (Wösten and Lugones, data not published). In fact, it has been suggested that by covering fungal aerial structures such as spores class I hydrophobins shield antigens in the cell wall, thereby protecting the fungal structure from the immune system [17,34]. These observations indicate that the use of hydrophobins in medical applications will probably not elicit immunogenic reactions.

To find a hydrophobin optimally suited to coat a particular implant the large variety of naturally occurring hydrophobins can be screened. Alternatively, hydrophobins can be modified by chemical cross-linking or genetic engineering. Recently, genetically engineered derivatives of the SC3 hydrophobin of *S. commune* were obtained by deleting 25 N-terminal amino acids (TrSC3) and/or fusing the cell binding domain of fibronectin (i.e. the RGD tripeptide) to the N-terminus of the mature hydrophobin (RGD-SC3 and RGD-TrSC3) [28]. These modifications did not affect gross properties of the hydrophobin but changed the surface characteristics of the hydrophilic side of the assemblage. The truncated forms of SC3 were less wettable than the full size hydrophobins. This reduced wettability can be explained by the absence of mannose residues. In contrast to SC3 that contains 16–22 mannose residues, TrSC3 is not glycosylated due to the absence of the O-glycosylation

sites contained in the stretch of deleted amino acids [35]. X-ray photon electron spectroscopy indicated that the mannose residues of SC3 are exposed at the hydrophilic side after assembly [36].

We here addressed whether hydrophobins are potentially cytotoxic and whether growth of cells on a hydrophobic solid can be improved by coating with (genetically engineered) hydrophobin. To assess cytotoxicity, up to $125 \mu\text{g ml}^{-1}$ monomeric or assembled SC3 or TrSC3 were added to the medium of 24 h-old fibroblast cultures. Since 1.5 mg of SC3 is sufficient to coat 1 m^2 of Teflon [20], the amount added to the medium would correspond to 1700 cm^2 of coated material. It thus represents a significant amount when extrapolated to an in vivo system. At all concentrations used, SC3 and TrSC3 did neither affect cell morphology and cell lysis nor confluence and cell numbers. Only at the highest concentration of monomeric TrSC3 an effect on cell numbers was observed after 24 h, which is not alarming since the assembled form and not the monomers of the hydrophobin are of interest for applications. From this we conclude that SC3 and TrSC3 are not toxic to fibroblasts. Yet, mitochondrial activity of cells as assessed by the MTT test was affected by the presence of SC3 and TrSC3. Mitochondrial activities were reduced more than 30% at the highest concentration of SC3 after 24 h and of TrSC3 after 72 h of growth.

There is an apparent discrepancy between cell numbers and the mitochondrial activity. This discrepancy has been observed in other studies related to biomaterials as well. It has been shown that mitochondrial activity as assessed by the MTT test can be quite variable between and within different experiments [37]. MTT activity of human skin fibroblasts grown on fibronectin-preadsorbed silicon rubber was slightly higher than that of cells grown on bare silicon, and were lower than that of cells grown on tissue culture quality polystyrene, the latter being used as a positive control [33]. It was thus concluded that a higher MTT conversion is not necessarily equivalent to a better biocompatibility. The slightly higher metabolic activity on fibronectin-preadsorbed silicon rubber surfaces, for example, could be explained by the extra energy required to re-arrange the fibronectin [38]. In the case of the fibroblast cultures in the presence of hydrophobins it cannot be excluded that the hydrophobins interact with the cells in some unknown way and thereby suppress metabolic activity. This suppression, however,

does not result in declined cell numbers or aberrant morphology. Therefore, the depression in MTT activity in fibroblasts exposed to the monomeric or assembled hydrophobin most probably is not a cytotoxic response. In order to confirm this, we will investigate whether other cell functions are affected as well.

Fibroblasts were grown in cell cultures on hydrophobin-coated Teflon to assess whether genetic engineering of the N-terminus of SC3 can be used to improve cell adhesion. Cells hardly grew on bare Teflon or Teflon coated with SC3 and were rounded and not spread flat as observed in wells without Teflon. Fusion of the RGD peptide to SC3 improved cell adhesion. Whether this is due to a slightly decreased wettability or due to interaction of integrins of fibroblasts with the RGD peptide remains to be established. A similar increase in cell numbers was observed with TrSC3. However, morphology of cells exposed to TrSC3 was more similar to that of cells grown in the absence of Teflon and was thus improved compared to coating with RGD-SC3. The improvement of cell adhesion by TrSC3 compared to SC3 may be due to decreased wettability of the former hydrophobin, being closer to the optimal water contact angle range with respect to cell adhesion and spreading [39]. In agreement, the SC4 hydrophobin of *S. commune* promoted cell growth similar to TrSC3. The wettability of the hydrophilic side of these hydrophobins is similar although they share 45% amino-acid identity only. No further improvement of cell adhesion was observed for RGD-TrSC3 as far as cell numbers are concerned. However, like in the experiments where hydrophobin was added to the medium, mitochondrial activity was decreased in the direct contact type of this test system. The fact that cell growth and cell morphology were not adversely affected by direct contact to the hydrophobin also indicates that cytotoxicity most likely is not involved in the observed decreased MTT activity.

5. Conclusions

Combining the results obtained with cells exposed to hydrophobins freely occurring in the medium or assembled at the hydrophobic surface of Teflon, it can be concluded that these proteins are not cytotoxic to fibroblasts and that hydrophobins can be genetically modified to better sustain cell adhesion. These promising data are a first step towards the assessment of biocompatibility.

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