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Basic Investigation

Synergistic Effects of a Novel Nanoporous Stent Coating and Tacrolimus on Intima Proliferation in Rabbits

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To overcome the problem of in-stent restenosis, the concept of local delivery of antiproliferative or immunosuppressive drugs has been introduced into interventional cardiology. Local drug delivery can be achieved by drug-eluting stents coated with polymer surfaces used for controlled drug release. However, several polymer coatings have shown an induction of inflammatory response and increased neointima formation. In the present study, the effect of a new inorganic ceramic nanoporous aluminum oxide (Al_2O_3) coating on neointima proliferation and its suitability as a carrier for the immunosuppressive drug tacrolimus have been investigated. 316 L stainless steel coronary stents were coated with a 500 nm thin nanoporous aluminum oxide layer. This ceramic nanolayer was used as a carrier for tacrolimus. Bare stents (n = 6), ceramic coated stents (n = 6), and ceramic coated stents loaded with 60 (n = 7) and 120 μg (n = 6) tacrolimus were implanted in the common carotid artery of New Zealand rabbits. The ceramic coating caused no significant reduction of neointimal thickness after 28 days. Loading the ceramic stents with tacrolimus led to a significant reduction of neointima thickness by 52% for 60 μg ($P = 0.047$) and 56% for 120 μg ($P = 0.036$) as compared to the bare stents. The ceramic coating alone as well as in combination with tacrolimus led to a reduced infiltration of lymphocytes and macrophages in the intima in response to stent implantation. Ceramic coating of coronary stents with a nanoporous layer of aluminum oxide in combination with tacrolimus resulted in a significant reduction in neointima formation and inflammatory response. The synergistic effects of the ceramic coating and tacrolimus suggest that this new approach may have a high potential to translate into clinical benefit. *Catheter Cardiovasc Interv 2003;60:399–407.*

Key words: neointima; restenosis; FK506; tacrolimus; stent coating

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INTRODUCTION

Although several studies have shown the superiority of coronary stent implantation as compared to conventional balloon angioplasty, restenosis remains a clinical problem [1,2]. To overcome this major drawback, the concept of drug-eluting stents has been introduced into interventional cardiology [3]. Using this approach, drugs are either chemically bonded onto the surface of the stent or the drug is trapped in polymer films that allow a controlled drug release. However, inflammatory reactions often induced by polymers are a serious limitation of this approach. Thus, the potentially favorable effects of antiproliferative, immunosuppressive, and anti-inflammatory drugs might be counteracted by the response to the polymer [4]. Although inorganic coatings have revealed promising results as so-called barrier coatings [5], they have not yet been used as coatings for drug release.

Inflammation is one of the major determinants of neointima formation after stent implantation [6]. A close correlation exists between the degree of inflammatory reaction and the extent of neointimal thickness [7]. Tacrolimus, also known as FK506, is a potent immunosuppressive drug with proven inhibitory activity on human vascular smooth muscle cells with an IC_{50} value of approximately 0.5 μM [8,9]. Tacrolimus exerts profound inhibitory effects on T-lymphocyte activation by binding specifically to FK506 binding protein 12 (FKBP12). The resulting tacrolimus-FKBP12 complex inhibits the cytoplasmatic phosphatase calcineurin, which activates transcription factor NFAT. After dephosphorylation [10], NFAT translocates into the nucleus and activates several cytokine genes involved in immune response and inflammation [11]. By inhibition of calcineurin via binding to FKBP12, tacrolimus inhibits the expression of IL-2, IL-3, IL-4, IL-5, IFNγ, GM-CSF, and TNFα, with IC_{50} values between 0.06 and 1.22 nM, leading to reduced immune response and inflammation [12,13]. In addition, tacrolimus exerts an anti-inflammatory and immunosuppressive effect through stimulation of TGFβ expression [14]. Tacrolimus can also inhibit apoptosis via inhibition of FasL release with high potency [15]. Recent evidence from $FKBP12^{-/-}$ knockout mice suggests that the FKBP12/FK506 complex is also affecting the expression of cell cycle proteins. It was found that p21 is upregulated in $FKBP12^{-/-}$ knockout mice, which in turn leads to G1 cell cycle arrest [16]. It is speculated that this mechanism is responsible for the antiproliferative effect observed in vascular smooth muscle cells [9,10]. Finally, studies on human specimens from restenotic lesions showed a clear upregulation of the expression of FKBP12, which further supports the use of tacrolimus for prevention of in-stent restenosis [17].

The aim of the present study was to evaluate a new nanoporous ceramic stent coating for drug delivery, to study the effect of this new ceramic coating on intima proliferation, and to explore a potential synergistic effect of the ceramic coating and tacrolimus on neointima proliferation.

MATERIALS AND METHODS

Ceramic-Coated Stents and Ceramic Drug-Coated Stents

316 L stents (Jomed, Rangendingen, Germany) were used for in vitro studies and implantation. The stents had a length of 16 mm, a surface area of 91.81 mm^2, and a strut diameter of 0.12 mm. Stents were coated in a two-step process. First, stents were coated inside and outside with a thin layer of aluminum using a specially designed physical vapor deposition process (anodic arc discharge, involving evaporation and ionization of metal vapor). This process ensured excellent adhesion of the coating to the base metal. In the second step, the metallic layer was electrochemically converted into a nanoporous ceramic (alumina/aluminum oxide/Al_2O_3) using a bath of 2% oxalic acid in water −0°C. The pore size was adjusted by this process [18]. When using pores between 5 and 15 nm, the pore density was approximately 10^9 pores cm^{-2} (Fig. 1). Drug coating was achieved by dipping the stents into a defined solution of 3 mg tacrolimus (Fujisawa Pharmaceutical, Japan) in 1 ml methanol with subsequent drying steps. Drug loading has been verified in control stents by solving tacrolimus from the stent coating using methanol and subsequent analysis by high-pressure liquid chromatography (HPLC; Gynkotek High Precision Pump Model 480) with a Hypersil BDS C18 3μ column. Two different tacrolimus loadings on ceramic-coated stents were tested. Mean tacrolimus loading was 60 ± 10 μg in group 1 and 120 ± 8 μg in group 2.

Raster Electron Microscopy

Electron microscopy was performed on ceramic-coated stents using a field emission scanning electron microscope (Philips XL 30 SFEG, Phillips, Eindhoven, The Netherlands) without conductive coating before imaging. The accelerating voltage has been set to less than 2 kV in order to minimize blunting effects of the ceramic surface. Stent deformation was assessed using finite-element calculation [19].

In Vitro Drug Release

In vitro drug release has been determined by immersing drug-eluting stents (60 μg) in 1.5 ml of distilled water in a glass vial for time periods of 24 hr. After this period, the stents were removed and 1.5 ml of methanol

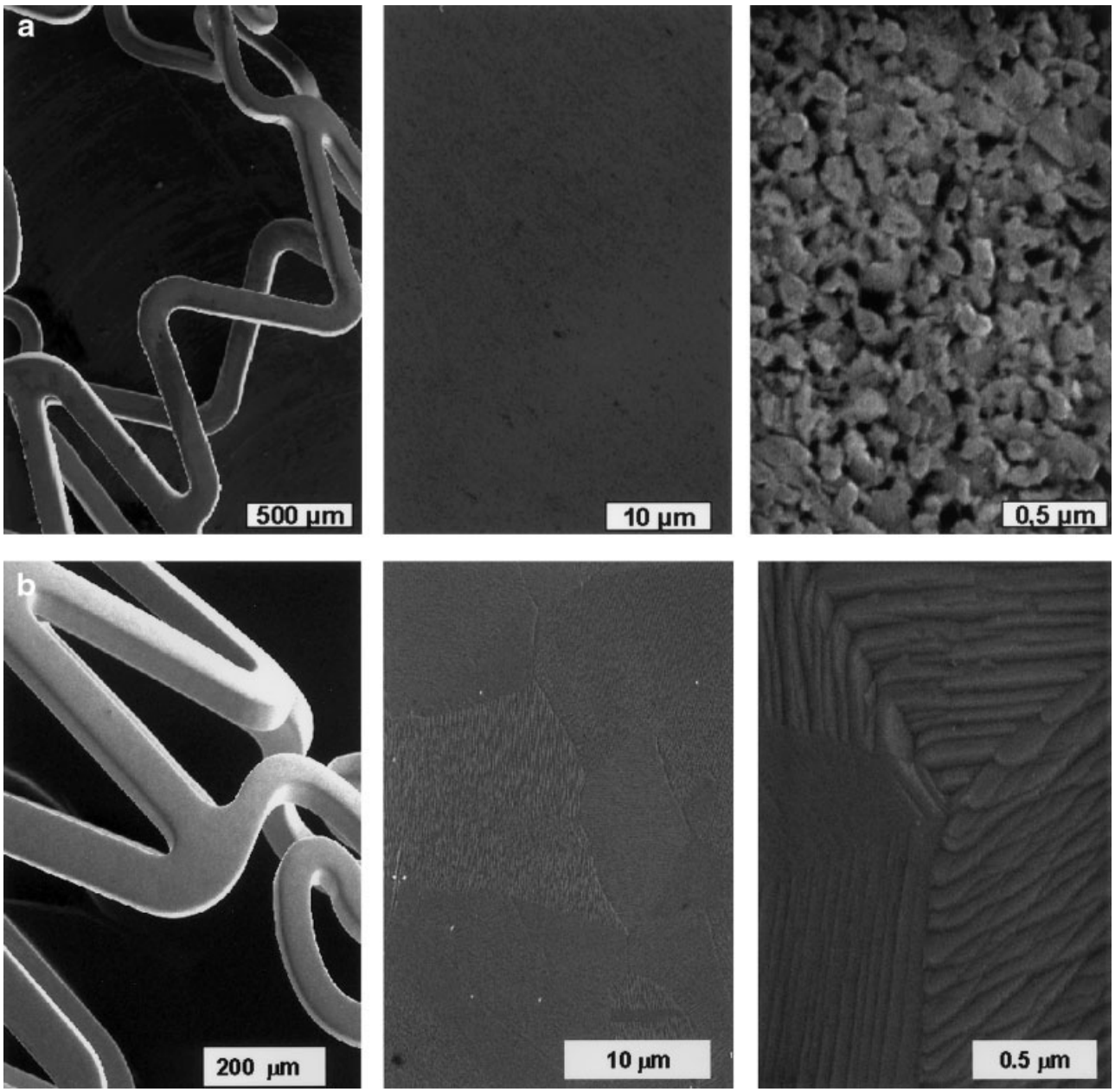

Fig. 1. Scanning electron microscopy image of a coated stent (a) and a bare stent (b). Despite the porous nature of the coating, the stent has a smooth surface.

was added to the glass vial. Drug released was determined by HPLC as described above. This procedure was repeated until the drug level reached the limit of detection, which is less than 0.5 µg/l.

Animals and Stent Implantation

New Zealand rabbits of either sex (3–4 kg) were used for the present study. Rabbits were given normal rabbit chow (Norlin 20ZH5, Germany). Acetylsalicylic acid was added to the drinking water in order to obtain an uptake of about 1 mg/kg body weight from day 1 until explantation. General anesthesia was done by weight-adapted intramuscular injection of ketamine (35 mg/kg Ketanest; Parke-Davis, Berlin, Germany) in combination with xylazine (5 mg/kg Rompun; Bayer, Leverkusen, Germany).

After exposing the common external and internal carotid artery with their side branches, a sheath (Braun Melsungen, Germany) was inserted in either the lingual or the facial artery. A 2 mm balloon catheter (Jomed)

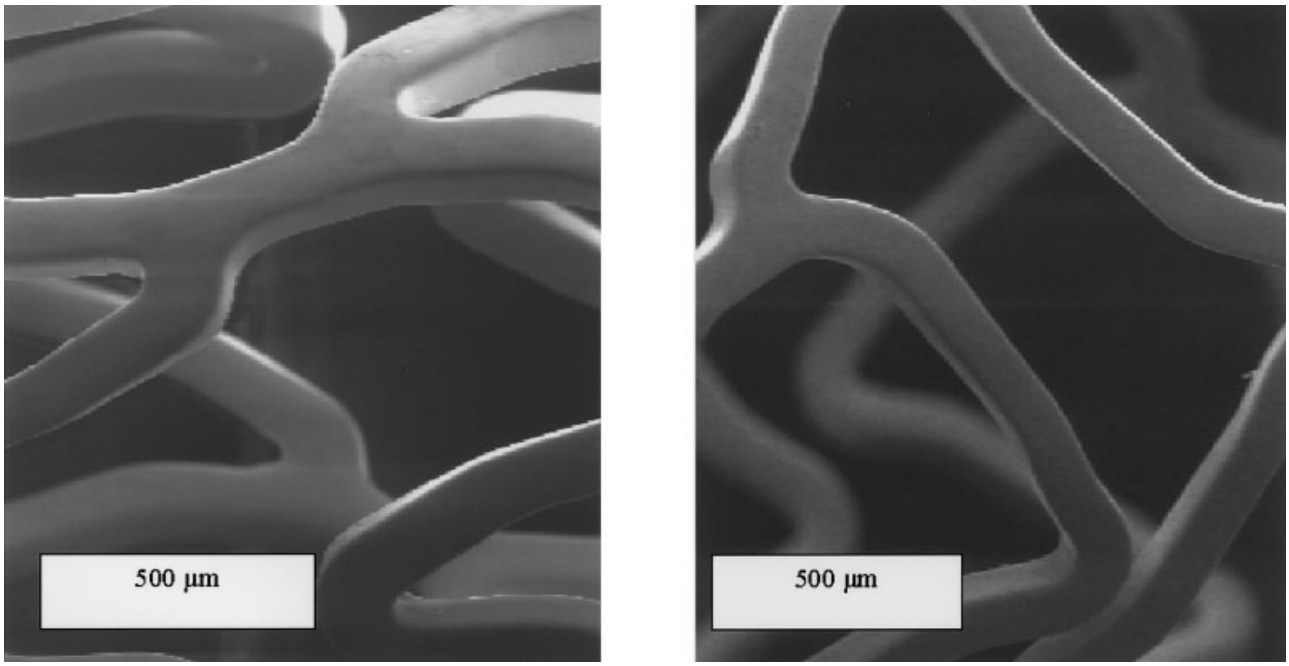

Fig. 2. Scanning electron microscopy image of a coated stent surface prior to (left) and after (right) dilatation. No delamination of the alumina coating has been observed.

with the coaxially mounted stent (Jomed) was inserted through the sheath until the stent was positioned in the common carotid artery. The stent was deployed by inflating the balloon with a pressure of 12 bar for 10 sec. The diameter of the common carotid artery was measured before and after stent implantation. Housing of the animals and all procedures were carried out according to the German Animal Welfare Legislation.

Study Design

Four different stents were implanted: group A (n = 6), bare stainless steel stents; group B (n = 6), stainless steel stents with ceramic coating; group C (n = 7), stainless steel stents with ceramic coating and low-dose tacrolimus loading (60 μg); group D (n = 6), stainless steel stents with ceramic coating and high-dose tacrolimus loading (120 μg).

Histology

Animals were sacrificed on day 28 and stented segments of the arteries were fixed in 4% buffered formalin. The stents were divided into two parts and either embedded in paraffin or acrylate. Acrylate sections were stained in toluidin-blue and used for morphometric evaluation. Morphometric analysis was done by measuring neointima thickness between stent struts in two sections. Hematoxylin-eosin and elastica van Gieson stainings were performed in paraffin sections. Inflammatory infiltrates close to the stent struts were determined using a semiquantitative scoring system per 400× field for lymphocytes (0, no cells; 1, ≤ 5 cells; 2, > 5 cells), histiocytes (0, no cells; 1, ≤ 10 cells; 2, > 10 cells), and granulocytes (0, no cells; 1, ≤ 5 cells; 2, > 5 cells). The neointima composition was assessed with respect to the predominance of cellular components or extracellular matrix. Local toxic effects were assumed when necrotic tissue appeared in the vicinity of the struts. The injury score was determined according to Schwartz et al. [20]

Drug Monitoring

After stent implantation, tacrolimus blood levels were determined using the IMx tacrolimus II assay (Abbott Laboratories, Chicago, IL). Whole blood levels were determined before implantation and at 1, 8, 24, and 48 hr after implantation. The detection limit of the assay in healthy humans is about 1.5 ng/ml. The target trough level in clinical liver transplantation is in the range of 10–15 ng/ml [21]. Tacrolimus exhibits an average $t_{1/2}$ of ~ 12 hr [22].

Statistical Analysis

Data are presented as mean ± SD. Continuous variables were compared using ANOVA with posthoc testing. P values of ≤ 0.05 were considered statistically significant. Data analysis was performed using SPSS (version 8.0; SPSS, Chicago, IL).

RESULTS

Electron Microscopy

Coated and uncoated stents were examined by electron microscopy (Figs. 1 and 2). The homogeneity of the coating was indistinguishable between the inner and outer surface of the stent. During dilation of the stents up to a diameter of 3.5 mm, a distinct plastic deformation of the base material occurred. It reached values up to 30% according to finite-element calculations. As shown in Figure 2, even under these conditions the coating adhered to the substrate. No delamination of the coating was observed even at higher magnifications. At higher magnification, the entrances of the pores could be observed as small black dots on the surface of the coating (Fig. 1a).

In Vitro and In Vivo Drug Release

In vitro drug release was studied using three ceramic stents loaded with 60 μg tacrolimus. Drug eluting showed a logarithmic distribution. After 96 hr, 73% ± 4.6% of the drug was eluted and in the following time period no further drug release was detectable (Fig. 3a).

In vivo drug release was investigated in six animals with ceramic-coated stents and 120 μg tacrolimus loading. Drug monitoring showed that tacrolimus blood concentration peaked 1 hr after implantation with 12.2 ± 4.7 ng/ml (range, 8.2–14.3 ng/ml). Within the first 48 hr after implantation, systemic levels of tacrolimus continuously decreased and reached the detection limit of the assay (Fig. 3b). Systemic concentration never exceeded the therapeutic range as defined for liver-grafted patients (15 ng/ml).

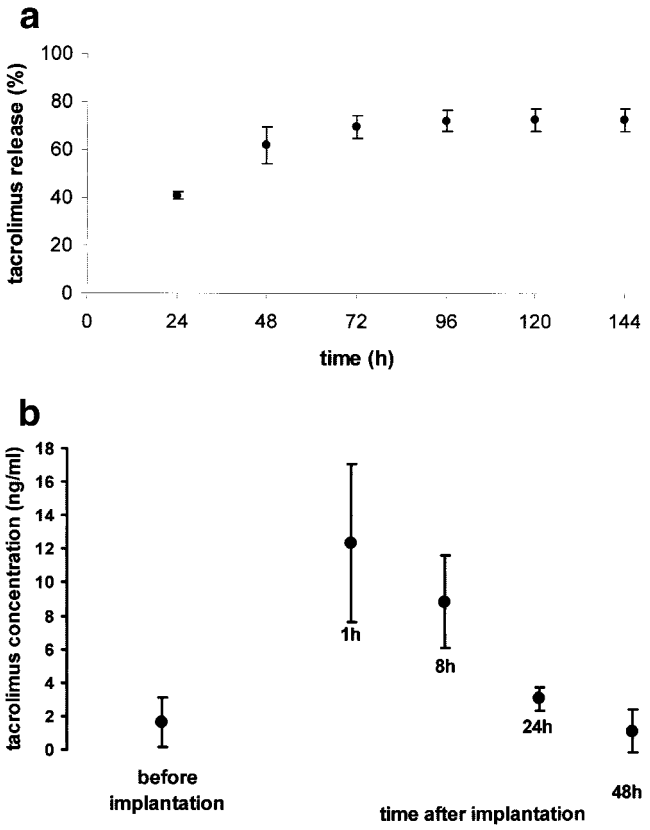

Fig. 3. a: In vitro drug release. Cumulative tacrolimus release within the first 144 hr. After 72 hr, about 75% of the loaded 60 μg tacrolimus has been eluted. About 25% is still trapped in the nanoporous Al_2O_3 coating. b: In vivo drug release. Time course of whole blood tacrolimus concentration after stent implantation in the carotid arteries of rabbits (n = 6).

Morphometry

Twenty-eight stents were implanted in 28 rabbits. Three animals died from bleeding into the bladder after heparin. Vessel diameter ratio before and after stent implantation ranged from 1.1 to 1.3 and was similar in all three groups. No stent thrombosis occurred in any of the treated animals. Ceramic coating caused no significant reduction of neointima thickness as compared to bare stents (111 ± 64 vs. 64 ± 21 μm; $P = 0.16$). A significant reduction of intima thickness occurred after loading the ceramic coating with 60 μg (53 ± 23 μm; $P = 0.047$ vs. bare stent) and 120 μg (49 ± 26 μm; $P = 0.036$ vs. bare stent) tacrolimus. The difference in neointima thickness in the two treatment groups did not reach statistical significance (Fig. 4).

The media thickness after 28 days did not differ in the bare stent and the ceramic-coated group (bare stent, 47 ± 9 μm; bare stent plus Al_2O_3, 54 ± 14 μm; $P = 0.23$). However, vessels with tacrolimus-loaded stents showed a significantly thicker media at the time of explantation than those with bare stents. This effect was not dose-dependent. There was no difference for the internal elastic area between the different groups. For details of conventional measures, see Table I.

Histology

Vessels subjected to stenting with any of the ceramic-coated stents displayed a rather mild inflammatory infiltrate with macrophages and lymphocytes in the neointima compared to the bare stents. Semiquantitative analysis revealed an additive effect in vessels stented with tacrolimus-loaded devices (Fig. 5).

In no section of either group could granulocytes be observed in light microscopic analysis. Giant cells, which are typical for foreign-body reactions, were observed in 5 out of 23 sections. In two sections of the ceramic group, in two sections of the ceramic group plus 60 μg tacrolimus, and in one section of the ceramic group plus 120 μg tacrolimus were giant cells present. Interestingly, in all sections from the ceramic group but not in the sections taken from the bare stents or the two

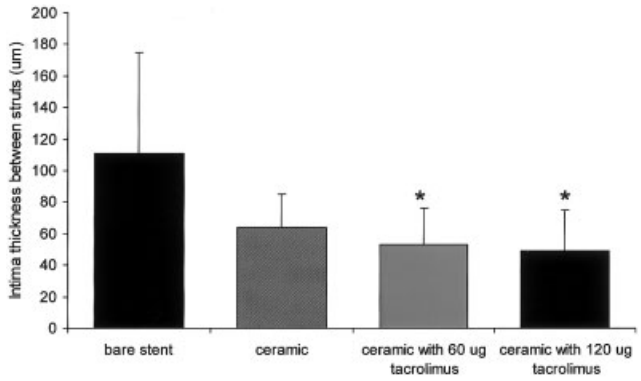

Fig. 4. Neointimal thickness 28 days after deployment of bare stents, stents with Al_2O_3 coating, stents with Al_2O_3 coating with 60 μg tacrolimus loading, and stents with Al_2O_3 coating with 120 μg tacrolimus loading. Intima thickness was measured between stent struts. Asterisk indicates $P < 0.05$.

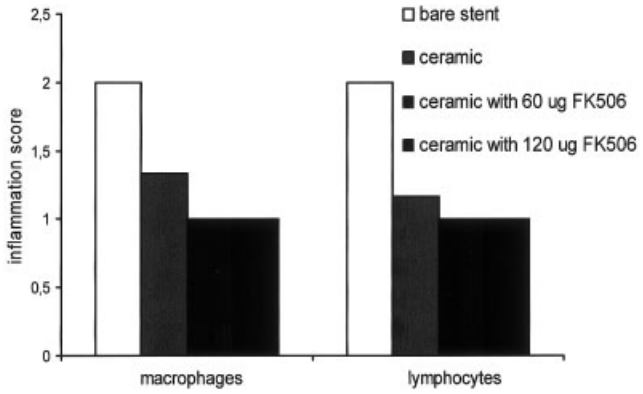

Fig. 5. Semiquantitative analysis of inflammatory cells close to the stent struts per 400× field. Lymphocytes (0, no cells; 1, ≤ 5 cells; 2, > 5 cells) and histiocytes (0, no cells; 1, ≤ 10 cells; 2, > 10 cells).

ceramic groups loaded with tacrolimus have foam cells been detected (Fig. 6b).

Analysis of the composition of the neointima revealed that all but 1 of the 12 stents loaded with tacrolimus (60 or 120 μg) revealed an intima primarily composed out of extracellular matrix. Only in one vessel was the predominant component smooth muscle cells. Similar to the tacrolimus groups in the ceramic group, the main component of all neointima specimens was extracellular matrix. In the bare stent group, in five animals extracellular matrix and in only one animal smooth muscle cells were the predominant component.

DISCUSSION

The present study presents preliminary results using a new nanoporous stent coating and the immunosuppressive drug tacrolimus for stent coating. The major findings of this study are that ceramic coating with nanoporous alumina is a suitable technology for stent coating; this coating serves as a suitable carrier for the immunosuppressive drug tacrolimus; and the ceramic coating in combination with tacrolimus leads to a significant inhibition of intima proliferation.

Ceramic Stent Coating

There are many in vivo animal trials investigating the biocompatibility of polymers [23,24]. Polymer films have been evaluated as direct surface coatings and as carriers of biologically active compounds. When nonerodable matrices are used, drug delivery is achieved through sustained release of the drug by diffusion through the porous matrix [25]. However, polymeric coatings are often associated with an inflammatory response [4], which is assumed to be one of the triggers for neointimal proliferation. In contrast, experimental and clinical studies using stents with inorganic coatings have shown promising results [5,26]. This favorable effect might be caused in part by the barrier effect of these coatings, which leads to a reduction of metal ion release [27]. However, the surfaces of inorganic stent coatings are usually not suitable for drug loading. In the present study, 316 L stainless steel stents were coated with a new nanoporous aluminum oxide layer. This ceramic coating was specially designed for drug loading and contains pores with a diameter between 5 and 15 nm. The morphometric and histological data obtained in the present study indicate that the nanoporous ceramic coating has a favorable tissue compatibility in terms of neointima formation and inflammatory response. This might be related to the chemical stability of dense alumina ceramics in physiological environments [28]. Especially when used as porous alumina ceramic, no scare tissue formation or inflammation occurs [29]. In addition, the porous nature of the ceramic coating is not accompanied by an increase in technical roughness of the stent surface, as demonstrated by raster electron microscopy (Figs. 1 and 2). This might be an important characteristic as experiments comparing differently processed stents from the same material suggest that a smooth surface reduces neointima formation [30].

The favorable effect of the applied ceramic coating might be related to the thin diameter of only 300–500 nm, which added only 2.5% to the original diameter of the struts. A recent clinical study convincingly pointed out that the use of thinner struts is associated with a significant reduction of angiographic restenosis after coronary artery stenting [31]. These results suggest that stent coatings should not substantially enhance strut thickness as this would promote intima proliferation and would

TABLE I. Data of Measures

	Bare stent	Ceramic	Ceramic with 60 μg tacrolimus	Ceramic with 120 μg tacrolimus
Lumen area (mm^2)	2.28 ± 0.16	2.6 ± 0.25	2.6 ± 0.09	2.47 ± 0.13
Intima area (mm^2)	1.0 ± 0.25	0.79 ± 0.08	0.72 ± 0.10[a]	0.7 ± 0.10[a]
Internal elastic area (mm^2)	3.28 ± 0.22	3.38 ± 0.27	3.35 ± 0.17	3.25 ± 0.07
Media thickness (μm)	47 ± 9	54 ± 14	72 ± 20[a]	71 ± 11[a]
Injury score	0.2	0.25	0.10	0.11

[a]$P < 0.05$ as compared to bare stent.

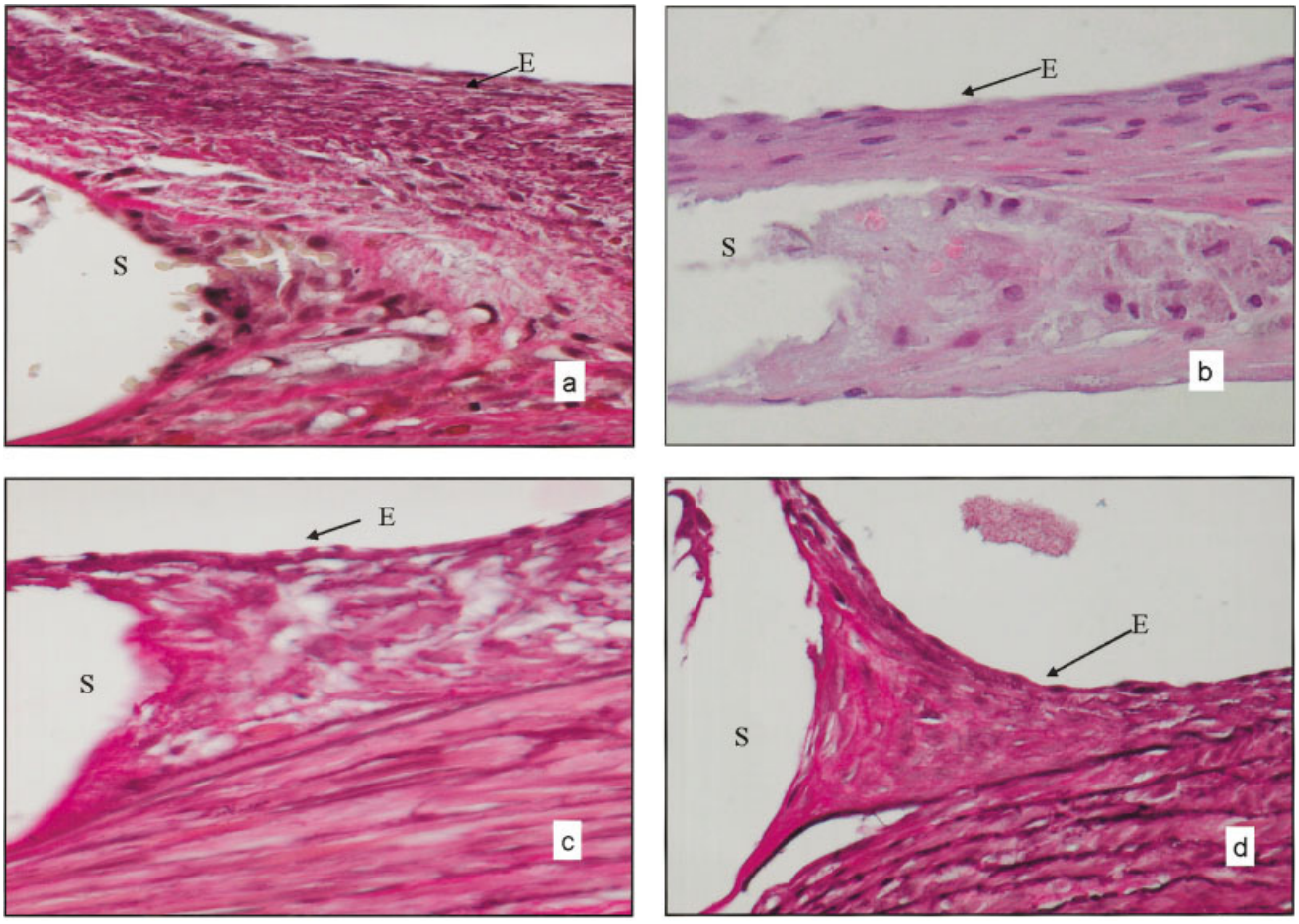

Fig. 6. Pictures demonstrating inflammatory cells close to the stent struts. a: Bare stents. b: Stents with Al_2O_3 coating. c: Stents with Al_2O_3 coating plus 60 μg tacrolimus loading. d: Stents with Al_2O_3 coating plus 120 μg tacrolimus loading.

counteract the favorable effect of restenosis-inhibiting drugs.

Tacrolimus for Inhibition of Neointima Formation

During the last years, growing scientific interest has been focused on local drug delivery for preventing restenosis after coronary intervention. Several drugs with different molecular modes of action have been proven effective to prevent restenosis in animal models [32–36]. Encouraging clinical results using this techniques have been published recently [3,37]. Tacrolimus (FK506) is a potent immunosuppressive drug, which has been widely evaluated in transplant patients [38]. Tacrolimus exerts profound inhibitory effects on T-lymphocyte activation by binding specifically to FK506 binding protein 12 (FKBP12) in the cytoplasm. The tacrolimus-FKBP12 complex inhibits calcineurin, which interrupts signal transduction pathways in T-cells [39]. Beside these anti-

inflammatory characteristics, tacrolimus also inhibits human vascular smooth muscle cell growth [9].

The present study demonstrates that ceramic-coated stents are suitable for loading and release of tacrolimus. The release kinetics indicate a 75% delivery of tacrolimus from the stent surface and the nanoporous coating over 2–3 days. The remaining 25% might be trapped in the porous architecture of the coating and might be released under the detection level of the used HPLC. Systemic blood levels exceeding the therapeutic range used in liver transplantation were not observed, so that the chance for patients to experience side effects might be rather low.

The morphometric data demonstrate that stents loaded with tacrolimus caused around 50% reduction of neointima thickness compared to bare stents. A similar degree of intima reduction was reported with other drugs [32]. However, the reduced infiltration of macrophages and T-cells in the intima provides additional evidence for the activity of tacrolimus in the stented arterial segments. Macrophages [40] and lymphocytes [41] are claimed to play a key role in the cascade of inflammation after stent implantation and promote the formation of neointima. The reduced infiltration of macrophages and T-cells might be attributed to the strong inhibitory effect of tacrolimus on the release of IL-2, IFNγ, and other cytokines [12].

The slight but significant increase of media thickness is probably due to the tacrolimus-mediated stimulation of TGFβ release [14]. This effect may translate into increased clinical safety, since the media might play a crucial role in the process of malapposition.

Study Limitations

The rabbit model is an accepted model for investigating the process of restenosis after stent implantation. However, a single animal model does not sufficiently reflect the complex pathomechanism in human atherosclerotic vessels. Interpretation of the results has to be done with caution, especially when considering a potential transfer into clinical settings, in which atherosclerotic human vessels are targeted. Further studies will have to investigate whether any of the two different doses of drug loaded on the stents have a sufficient potential to inhibit neointima formation in humans. Atherosclerotic human coronary plaques contain a considerable amount of inflammatory cells already prior to stent implantation.

In addition, studies will have to investigate whether the alumina coating alone and in combination with tacrolimus exhibit good biocompatibility in the long run. In this context, experiments will have to assess the issue on time course and pattern of reendothelialization as a delay of endothelial covering of a stent can cause severe complications.

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