A Novel Drug-Eluting Stent Coated With an Integrin-Binding Cyclic Arg-Gly-Asp Peptide Inhibits Neointimal Hyperplasia by Recruiting Endothelial Progenitor Cells

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OBJECTIVES

Novel stents loaded with an integrin-binding cyclic Arg-Gly-Asp peptide (cRGD) were analyzed for their potential to limit coronary neointima formation and to accelerate endothelialization by attracting endothelial progenitor cells (EPCs).

BACKGROUND

Re-endothelialization is important for healing after arterial injury. Effects of cRGD on EPC number, recruitment in flow, and invasion were analyzed in vitro. A durable polymer coating containing 67 µg cRGD per stent was developed for Guidant Tetra stents. Twelve cRGD-loaded polymer, 12 unloaded polymer, and 12 bare metal stents were deployed in porcine coronary arteries. Quantification of cRGD in peri-stent tissue was established by high-performance liquid chromatography (HPLC) and mass spectrometry (MS). Histomorphometry and immunostaining were performed after 4 and 12 weeks. Recruitment of labeled porcine EPCs was assessed 7 days after intracoronary infusion.

METHODS

The cRGD clearly supported the outgrowth, recruitment, and migration of EPCs in vitro. At 4 weeks, there was no difference for mean neointimal area and percent area stenosis in the cRGD-loaded, polymer, or bare metal stent group. At 12 weeks, neointimal area (2.2 ± 0.3 mm²) and percent area stenosis (33 ± 5%) were significantly reduced compared with polymer stents (3.8 ± 0.4 mm², 54 ± 6%; p = 0.010) or bare metal stents (3.8 ± 0.3 mm², 53 ± 3%; p < 0.001). The HPLC/MS confirmed cRGD tissue levels of 1 to 3 µg/stent at 4 weeks, whereas cRGD was not detectable at 12 weeks. Staining for CD34 and scanning electron microscopy indicated enhanced endothelial coverage on cRGD-loaded stents at 4 weeks associated with a significant increase in the early recruitment of infused EPCs.

RESULTS

The introduction of first-generation drug-eluting stents has clearly reduced the incidence of in-stent restenosis (ISR) after percutaneous coronary intervention, which has represented the Achilles heel of interventional cardiology for more than a decade. Several multi-center clinical trials have shown the superiority of drug-eluting stents compared with bare metal stents to reduce major cardiac events after percutaneous coronary intervention (1,2). Based on these data, the frequency of angiographic restenosis for the currently available drug-eluting stents range from 8% to 15% depending on the case or lesion complexity (3). The remaining cases of ISR might be explained by the limitations of the drugs in use, i.e., sirolimus and paclitaxel, which mainly act by inhibition of smooth muscle cell (SMC) proliferation. Thus, ISR still constitutes a significant problem given the large volume of coronary interventions and expanding indications (4).

The pathophysiology of neointimal hyperplasia in ISR comprises complex interactions between cellular and acellular elements of the vessel wall and the blood (5). It has been shown that a functionally intact endothelium is a prerequisite for the inhibition of neointimal growth after percutaneous coronary intervention (6), and that endothelial progenitor cells (EPCs) can be recovered from a peripheral pool of mononuclear cells (7). The EPCs have been characterized
by the expression of stem cell (CD133, CD34) and endothelial cell markers (vascular endothelial growth factor [VEGF] receptor 2, CD31, VE-cadherin, von Willebrand factor), and by their ability to develop colony-forming units (8,9). The contribution of EPCs to re-endothelialization after vascular injury has recently been shown. For example, infusion of EPCs after vascular injury and their mobilization and incorporation after statin treatment significantly reduced neointimal growth (10,11); however, the knowledge about homing and differentiation of EPCs at sites of injury remains incomplete. In studies with hematopoietic stem cells, a crucial role in these processes has been ascribed to integrins (12), a family of transmembrane adhesion receptors, which regulate cell phenotype and function and display specific binding and signaling properties (13). In prior studies, expression of integrins with integrin-binding cyclic Arg-Gly-Asp peptide (cRGD) binding motifs has been shown on EPCs (10,12). The RGD sequence is recognized by a variety of integrins, including αvβ3, α5β1, αvβ1, αvβ5, and αIIbβ3III, and is found in a variety of extracellular matrix components. According to the stereochemistry of the sequence Arg-Gly-Asp-Xaa, the binding specificity to the integrins can be controlled (14).

Thus, the goal of this study was to explore the potential of a hydrophilic cRGD peptide with a higher affinity for the αvβ3- than the α5β1-integrin receptor (15) to reduce neointimal hyperplasia by facilitating recruitment of circulating EPCs and endothelialization. Hence, the capacity of the cRGD to effectively attract EPCs was evaluated using a newly developed polymer stent loaded with the cRGD. It should be analyzed whether local delivery of cRGD was capable of mediating the attraction of EPCs and enhanced coronary endothelialization, thus significantly reducing neointimal hyperplasia after stent implantation.

**METHODS**

**Isolation and culture of EPCs and SMCs.** Peripheral blood mononuclear cells were separated by density gradient centrifugation from porcine blood. Isolated peripheral blood mononuclear cells were seeded in culture flasks with different coatings: either 1% bovine serum albumin, or 10 μg/ml human fibronectin, or 1 μg/ml and 100 μg/ml cRGD peptide (cyclic GPenGRGDSPCA with Pen indicating penicillamine, enabling a cyclic structure by disulfide bond formation with the cysteine and increasing the conformational restraints on the ring structure [14], relative molecular weight: 948.05) in microvascular endothelial growth medium. After seven days, isolated cells were subjected to cytochemical analysis, study under flow conditions, or invasion experiments. The SMCs were isolated and cultured as described (16,17).

**Characterization and number of EPCs.** For uptake of 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine-labeled Ac-low-density lipoprotein (DiI-Ac-LDL), peripheral blood mononuclear cell–derived EPCs were incubated with DiI-Ac-LDL and fluoresceinisothiocyanat-labeled Ulex europaeus agglutinin I. The DiI-Ac-LDL/lectin double-positive cells were judged as EPCs, and the percentage of DiI-Ac-LDL/lectin double-positive cells was analyzed by flow cytometry.

**Analysis of EPC attachment under flow conditions.** The EPC adhesion on bovine serum albumine (BSA), fibronectin (10 μg/ml), or surface-immobilized cRGD-peptide (1 μg/ml or 100 μg/ml) was quantified under flow conditions using a parallel plate flow chamber as described (18,19). Suspensions of EPCs were perfused, and the number of EPCs firmly adherent by primary interaction with the different matrices was quantified.

**Invasion assay.** Invasion assays were performed in a modified Boyden chamber as described (16,17). Filters were coated with a reconstituted basement membrane, the lower compartment was filled with Dulbecco modified eagle medium with or without cRGD peptide (100 μg/ml), and SMCs or EPCs were placed in the upper compartment. After incubation, adherent cells were counted.

**Stent design and polymer coating.** A new polymer stent coating was developed and subsequently applied to the stents (stainless steel Multi-Link Guidant Tetra stent [Santa Clara, California], 3-mm diameter, 13-mm length, 5.15 μg cRGD/mm stent length). The polymer coating fulfilled the requirements for appropriate local drug delivery of the hydrophilic cRGD peptide. It consisted of an adherent, conformal, mechanically robust coating based on an aromatic polyetherurethaneurea with a soft segment of polytetramethyleneoxide and a hard segment of diphenylmethane disiocyanate and mixed diarnines (BioSpan SPU, Polymer Technology Group, Berkeley, California) covered by a control-release layer.

**In vitro and in vivo pharmacokinetic studies.** Cumulative cRGD release was measured after simulated use of peptide-loaded stents by high-performance liquid chromatography (HPLC) in vitro. For in vivo pharmacokinetics, stents were used 4 and 12 weeks after implantation. Adjacent tissue was dissected as described (20), pre-processed, and subjected to reverse-phase (RP)–HPLC and mass spectrometry (MS) analysis of cRGD.
Animal model. Twelve polymer-coated cRGD-loaded, 12 unloaded polymer, and 12 stainless steel bare metal stents were deployed into the right and left coronary arteries of 18 domestic pigs (9 pigs per time point; 2 stents per animal; 6 stents per group). The stents were implanted using an incomplete factorial design, thus allowing intraindividual and interindividual comparisons, and the pigs were randomly assigned to these treatment modalities. The stent-to-artery ratio was 1.1 to 1.2, with similar ratios in all groups. Pigs were maintained on 75 mg clopidogrel and 100 mg aspirin per day and killed after 4 or 12 weeks. Thus, 6 stents of each type were subjected to histology at each time point. In an additional group of pigs, histologic assessment was combined with intravascular ultrasound analysis at 4 and 12 weeks: 4 stents of each type were implanted into 6 pigs (3 pigs per time point; 2 stents per animal; 2 stents per group). Thus, in total, 8 stents of each type were implanted per time point and group. Further animals were instrumented for in vivo drug elution (6 cRGD-loaded and 6 unloaded polymer stents were individually implanted into 12 pigs; 3 stents per group and time point), EPC injection (4 stents of each type implanted into 4 pigs; 3 stents per animal), and scanning electron microscopy experiments (3 stents of each type implanted into 3 pigs; 3 stents per animal).

Intracoronary infusion of labeled EPCs. Isolated porcine EPCs were detached with Accutase (PAA Laboratories, Pasching, Austria) and labeled with cell tracker CM-DiI (1 μg/ml, Molecular Probes-Invitrogen, Karlsruhe, Germany). Labeled EPCs (3 × 10^7) were infused into coronary arteries immediately after stent implantation. After seven days, pigs were killed, stents were explanted, and after opening by a longitudinal cut, the luminal area was inspected by en face fluorescence microscopy. The number of DiI-labeled cells was determined in all microscopic fields of the complete stent surfaces and compared with unloaded polymer and bare metal control stents. The area with specific EPC-stent surfaces and compared with unloaded polymer and was determined in all microscopic fields of the complete fluorescence microscopy. The number of DiI-labeled cells by 163 concentrations of cRGD resulted in an increase in the positive, i.e., EPCs (Fig. 1 B). The coating with different controls without cRGD (Fig. 2A). No significant difference between EPCs cultured in 3% glutaraldehyde for at least 1 h. They were dehydrated in a graded acetone series (30, 50, 70, 90, 3 × 100%) and critical-point-dried in carbon dioxide. The samples were fixed on specific stubs and sputter-coated with gold (SCD 030, Balzers Union), then investigated in an ESEM XL 30 FEG (FEI-Philips, Kassel, Germany) in high-vacuum mode.

Statistical analysis. All results are expressed as mean ± SEM. For the in vitro experiments, statistical significance was evaluated using unpaired Student t test or analysis of variance followed by the Dunnett post-hoc test for more than two means. For histomorphometric measurements, the Kruskal–Wallis test was used to determine overall statistical significance, followed by the Mann-Whitney U test with Bonferroni correction for subsequent pairwise comparison if the overall significance was <0.05; p values < 0.05 were considered significant.

RESULTS

The effect of cRGD on the outgrowth of EPCs. To investigate the effect of surface-bound cRGD on the outgrowth of EPCs, the number and the morphology of cells bound to the peptide were determined in vitro. After seven days in culture, the occurrence of cells with a typical spindle-shaped phenotype could be observed (Fig. 1A). Adherent cells were harvested and identified as EPCs by DiI-Ac-LDL/lectin double staining using flow cytometry. More than 90% of adherent cells were identified as double-positive, i.e., EPCs (Fig. 1B). The coating with different concentrations of cRGD resulted in an increase in the number of EPCs by 163 ± 9% at 1 μg/ml (p = 0.007) and by 170 ± 11% at 100 μg/ml (p = 0.003), compared with controls without cRGD (Fig. 2A). No significant difference in cell number increase was found between EPCs cultured on cRGD or fibronectin (167 ± 10% vs. cRGD at 100 μg/ml, p = 0.765).

Recruitment of EPCs by cRGD under flow conditions. To determine the effect of cRGD on EPC recruitment under flow conditions, porcine EPCs were perfused at a shear rate of 3 dynes/cm² in a parallel-wall flow chamber. Although few EPCs displayed transient rolling interaction after initial tethering on cRGD, most EPCs underwent...
immediate arrest resulting in stable adhesion. Much weaker arrest was seen on BSA-coated plates (Fig. 2B). Coating with cRGD resulted in a significant increase of shear-resistant EPC arrest (159 ± 7% at 1 μg/ml, p = 0.04; 164 ± 8% at 100 μg/ml, p = 0.009; both vs. BSA control), which was comparable with fibronectin-mediated arrest (164 ± 9% vs. cRGD at 100 μg/ml, p = 0.814).

**Effects of cRGD on the invasive potential of EPCs and SMCs.** The potential of cRGD to act as a chemoattractant for EPCs or SMCs was determined using a modified Boyden chamber. The EPCs were used after distinct periods of culture (5 and 12 days). For both subgroups, cRGD significantly stimulated the invasion of EPCs (209 ± 35% of control at 5 days, p = 0.021, 355 ± 45% of control at 12 days, p = 0.0001) (Fig. 3A). In contrast, cRGD did not stimulate the invasive potential of SMCs (p = 0.196) (Fig. 3B).

**Stent characteristics and in vitro pharmacokinetics.** The HPLC analysis of cRGD enabled its detection with linearity across a range of concentrations (1 to 100 μg/ml). Stability of the peptide dissolved in phosphate-buffered saline was shown from 4°C to 37°C and for up to 10 days (data not shown). After incorporation of the peptide into the polymer matrix of the stents, electron microscopy showed complete surface integrity after crimping onto delivery system catheter, after passing through a guiding catheter, and after subsequent stent expansion in phosphate-buffered saline (simulated use) (Fig. 4A). In vitro drug release of cRGD-loaded stents (n = 3) showed an exponential release profile over at least 72 h. After 72 h, 52% of the cRGD load (35 of 67 μg) had been eluted, so that a continuous release thereafter can be assumed (Fig. 4B).

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**Figure 1.** Characterization and outgrowth of isolated porcine endothelial progenitor cells (EPCs). (A) Mononuclear cells were plated on culture dishes in the presence (1 μg/ml or 100 μg/ml, respectively) or absence of integrin-binding cyclic Arg-Gly-Asp peptide (cRGD) coating. Coating with cRGD provoked a significant dose-dependent increase of spindle-shaped cells compared with uncoated control experiments. Representative images from four isolations are shown, magnification 100×. (B) Flow cytometry analysis of adherent cells at day 7 of culture. Unstained cells served as a control condition. Analysis was performed by manual gating and fluorescence channel (FL)-1/FL-2 dot plot quadrant statistic. 1,1’-dioctadecyl-3,3,3’3’-tetramethylindocarbocyanine-labeled Ac-low-density lipoprotein/lectin double-positive cells in the upper right region were judged as EPCs. FITC = fluorescein isothiocyanate.

**Figure 2.** Recruitment of isolated endothelial progenitor cells (EPCs) on integrin-binding cyclic Arg-Gly-Asp peptide (cRGD) matrix. (A) After 7 days in culture, adherent cells were counted in multiple microscopic fields. Coating with cRGD (1 μg/ml or 100 μg/ml) resulted in a significant increase in EPC outgrowth compared with uncoated control experiments. The fibronectin coating served as a positive control condition (n = 4, *p < 0.05). (B) Coating with cRGD (1 μg/ml or 100 μg/ml) resulted in a significant increase in EPC arrest under flow conditions compared with uncoated control experiments. The fibronectin coating served as a positive control condition (n = 4, *p < 0.05). FN = fibronectin.
Release profiles were similar for unexpanded and expanded stents (data not shown). 

**In vivo pharmacokinetics.** Tissue levels of cRGD were analyzed by RP-HPLC and MS to confirm whether the observed biological effects correlated with cRGD levels in the adjacent vascular wall. A procedure was established allowing the detection of cRGD from complex tissue lysates. On optimization, it was possible to recover and detect cRGD spiked into 200 mg of tissue lysate. The RP-HPLC analysis of tissue lysates from unloaded stents did not yield any peaks at or near the elution volume of cRGD (Fig. 5A). Recovery of cRGD from the tissue surrounding the stent was determined and compared between 4 and 12 weeks. The RP-HPLC analysis showed that small amounts (1 to 3 μg) of a peptide peak with a molecular mass <3 kDa were detected in tissue lysates excised after 4 weeks (Fig. 5B). Externally added (“spiked”) cRGD eluted at the same volume, indicating that the recovered peptide was cRGD (Fig. 5B). Identity of recovered cRGD was finally confirmed by MS analysis (Figs. 5C and 5D). The mass of the isolated peptide species from tissue samples was 474.5 Da, and thus was identical to that of pure standard cRGD (M+2 mass of 474.8 ± 0.3 Da) (Fig. 5C). In addition, an identical mass was obtained when cRGD was spiked into a raw control tissue lysate that underwent an identical procedure (Fig. 5D). Thus, cRGD was efficiently released from stents into the adjacent tissue and measurable cRGD levels were present in the tissue after four weeks. In contrast, after 12 weeks, tissue lysates did not contain detectable cRGD (data not shown).

**Histomorphometric measurements.** All vessels were angiographically and histologically patent at all time points. Stent malapposition was not detectable in histologic specimens or by intravascular ultrasound examination after stent deployment (Fig. 6A). At 4 weeks, cross-sectional area stenosis was 35 ± 4%, 31 ± 2%, and 36 ± 4% for unloaded polymer, cRGD-loaded polymer, and bare-metal stents, respectively (p = 0.674). Mean neointimal areas were 2.5 ± 0.3 mm², 2.3 ± 0.2 mm², and 2.6 ± 0.2 mm² (p = 0.268), and neointimal thickness was 0.41 ± 0.05 mm, 0.31 ± 0.04 mm, and 0.42 ± 0.05 mm (p = 0.310); mean internal elastic lamina areas were 7.2 ± 0.2 mm², 6.9 ± 0.3 mm², and 7.3 ± 0.3 mm² (p = 0.462), and mean external elastic lamina areas were 8.2 ± 0.2 mm², 7.9 ± 0.3 mm², and 8.2 ± 0.3 mm² (p = 0.696) for unloaded polymer, cRGD-loaded polymer, and bare-metal stents, respectively. At 12 weeks, cross-sectional area stenosis of the cRGD-loaded stents was...
significantly lower (33 ± 5%) as compared with unloaded polymer or bare-metal stents (54 ± 6% and 53 ± 3%, p = 0.010 and p < 0.001, respectively), whereas no differences were observed between unloaded polymer and bare-metal stents (p = 0.744). The corresponding mean neointimal areas and neointimal thicknesses were 3.8 ± 0.4 mm², 2.2 ± 0.3 mm², and 3.8 ± 0.2 mm² (p < 0.001 for cRGD-loaded vs. unloaded and for cRGD-loaded vs. bare-metal, respectively; p = 0.657 for unloaded vs. bare-metal) and 0.67 ± 0.06 mm, 0.39 ± 0.05 mm, and 0.72 ± 0.05 mm (p = 0.006 for cRGD-loaded vs. unloaded, p = 0.001 for cRGD-loaded vs. bare-metal, and p = 0.295 for unloaded vs. bare-metal) for unloaded polymer, cRGD-loaded polymer, and bare-metal stents, respectively. Mean internal elastic lamina areas at 12 weeks were 7.4 ± 0.2 mm², 7.0 ± 0.1 mm², and 7.3 ± 0.2 mm² (p = 0.531), mean external elastic lamina areas were 8.4 ± 0.2 mm², 8.4 ± 0.1 mm², and 8.3 ± 0.2 mm² for unloaded polymer, cRGD-loaded, and bare-metal stents, respectively (p = 0.871). Injury scores did not differ among the groups. All histomorphometric data are summarized in Table 1.

**Histopathologic analysis.** Only mild infiltration with CIC was observed around the stent struts, indicating a sufficient biocompatibility of the polymer. Inflammation rates were similar in all three stent groups at 4 and 12 weeks (unloaded polymer, RGD-loaded polymer, and bare-metal stents at 4 weeks) and did not differ among the groups. All histologic scores were near zero (p = 0.687). The injury scores did not differ among the groups. All histomorphometric data are summarized in Table 1.
segments distant to the stent struts, no relevant inflammatory infiltration was seen. In contrast to sirolimus- and paclitaxel-eluting stents (21), no peri-stent fibrin deposition (a marker of stent-induced injury healing) was identified in any of the stent groups (data not shown). To evaluate the in vivo effects of cRGD coating on EPC recruitment, stent coverage by circulating progenitor cells was assessed by CD34 staining, electron microscopy, and the presence of DiI-labeled porcine EPCs injected into the coronary artery directly after stent implantation (Fig. 7). At 4 weeks, the cRGD-loaded stents were lined by $83 \pm 3$ CD34+ cells/field, whereas only $35 \pm 2$ and $36 \pm 3$ CD34+ cells/field were observed in the unloaded polymer and bare metal stent group ($p = 0.020$ for cRGD-loaded vs. unloaded stents, $p = 0.021$ for cRGD-loaded vs. bare-metal stents) (Figs. 7A and 7B). This was also illustrated by electron microscopy (Fig. 7C). At 12 weeks, stent coverage was complete in all groups without significant differences ($87 \pm 8$, $95 \pm 6$, or $93 \pm 3$ CD34+ cells/field for unloaded polymer, cRGD-loaded polymer, or bare metal stents, respectively; $p = 0.585$). Moreover, intracoronary injection of DiI-labeled EPCs at the time of stent placement showed their preferential recruitment to cRGD-loaded (fluorescent labeling: $1.3518 \pm 0.2257$ mm²/stent, $p = 0.027$ for cRGD vs. unloaded polymer stents, $p = 0.006$ for cRGD vs. bare metal stents; cell counting: $22.5 \pm 1.4\%$ of high-power fields containing DiI-labeled EPCs, $p = 0.037$ for cRGD vs. unloaded polymer stents, $p = 0.046$ for cRGD vs. bare metal stents) but not control stents (fluorescent labeling: $0.2419 \pm 0.1522$ and $0.2156 \pm 0.1802$ mm²/stent; cell counting: $2.5 \pm 0.3\%$ and $3.3 \pm 0.8\%$ positive high-power fields for unloaded polymer and bare-metal stents, respectively) (Figs. 7D and 7E).

**DISCUSSION**

The present study shows that a cyclic RGD peptide with αvβ3-integrin-binding capacities can: 1) stimulate outgrowth, shear-resistant recruitment, and invasion of EPCs,

**Table 1.** Histomorphometric Data

<table>
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<tr>
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<th>Unloaded Polymer Stents</th>
<th>cRGD-Loaded Polymer Stents</th>
<th>Bare-Metal Stents</th>
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<td>4 weeks (n = 8 per group)</td>
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<td>Percent cross-sectional area stenosis (%)</td>
<td>35 ± 4</td>
<td>31 ± 2</td>
<td>36 ± 4</td>
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<td>Lumen area (mm²)</td>
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<td>Neointimal area (mm²)</td>
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<td>Neointimal thickness (mm²)</td>
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<td>External elastic lamina area (mm²)</td>
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<td>Injury score</td>
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<td>12 weeks (n = 8 per group)</td>
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<tr>
<td>Percent cross-sectional area stenosis (%)</td>
<td>54 ± 6</td>
<td>33 ± 5</td>
<td>53 ± 3</td>
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<td>Lumen area (mm²)</td>
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<td>Neointimal area (mm²)</td>
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<td>Internal elastic lamina area (mm²)</td>
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<td>0.918 (NS)</td>
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*Statistically significant.

cRGD = integrin-binding cyclic Arg-Gly-Asp peptide; NS = not significant.
Figure 7. Effects on endothelialization. (A) Representative photomicrographs of endothelialization, analyzed by CD34 staining, of unloaded polymer, integrin-binding cyclic Arg-Gly-Asp peptide (cRGD)-loaded, or bare-metal peri-stent tissue sections after 4 and 12 weeks (magnification 400×). (B) Quantification of endothelialization of unloaded polymer, cRGD-loaded, or bare-metal persistent tissue sections. (C) Electron microscopy of cRGD stent surfaces at 4 weeks showed uniform and complete endothelial coverage (left), whereas analysis of control stents showed incomplete endothelial coverage in the interstrut region. (D) The cRGD coating (left) significantly increased endothelial progenitor cell (EPC) recruitment as compared with control stents (right, magnification 200×). (E) Quantification of 1,1-di-octadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI)-labeled EPCs by fluorescent image analysis (top) or cell counting (bottom).
2) reliably be incorporated into and released from a newly designed polymer stent coating, and thereby, 3) significantly inhibit the extent of neointimal hyperplasia by accelerating stent endothelialization.

Endothelial denudation is considered to be the primary injury after balloon angioplasty and stent implantation. When larger areas are denuded or endothelial recovery is delayed, a higher degree of intimal thickening ensues (22). Furthermore, it could be shown that conditioned serum of injured endothelial cells can stimulate proliferation and migration of SMCs (23). Several studies have also highlighted the importance of bone marrow-derived EPCs for the process of healing after vascular injury. For instance, the number of circulating EPCs is significantly increased after myocardial infarction (24). Evidence showing that EPCs can also be used as a therapeutic approach to accelerate re-endothelialization after injury has recently been provided (11). Treatment with statins and the inhibition of pro-migratory factors have implicated the recruitment of EPCs in accelerating endothelial recovery and reducing neointima (25). Similarly, accelerated re-endothelialization by local transfer of the VEGF-2 plasmid or by statin therapy has been shown to inhibit neointima formation likely by increasing the mobilization and incorporation of EPCs (26).

Nevertheless, the mechanisms underlying EPC recruitment (homing) to the site of injury remain to be elucidated. Although the blockade of the keratinocyte-derived chemokine seems to enhance neointimal formation by delaying endothelial recovery (22), this effect may in part be attributable to interference with the recruitment and incorporation of EPCs.

For this study, cRGD characterized by higher affinity for αvβ3 than α5β1 integrins (15) was incorporated into a specifically designed stent and evaluated for its ability to recruit EPCs and to reduce neointimal hyperplasia. For hematopoietic progenitor cells, earlier studies have suggested a role of integrins in supporting cell adhesion (27).

Although SMC outgrowth cells from peripheral blood have been shown to preferentially express β1 integrins binding to fibronectin (10), coating with cRGD seemed to attract predominantly CD34+ EPCs. The results presented herein would be consistent with the notion that EPCs express αvβ3 at higher levels than SMC progenitors. In vitro isolation and culture of EPCs on cRGD also resulted in an increased outgrowth of EPCs. In addition, the effect of cRGD in promoting EPC adhesion could also be evidenced in a model simulating flow conditions in human arteries (28).

Finally, an established Boyden chamber model showed that cRGD does not solely stimulate recruitment of EPCs to cRGD-coated surfaces, but also acts as a chemoattractant for EPCs to induce their transmigration. Notably, nonquiescent SMCs showed a lesser responsiveness to cRGD in a side-by-side analysis of their invasive potential. Thus, this indeed seems to be attributable to a higher expression of αvβ3 integrin on EPCs as compared with SMC progenitors (29).

Moreover, these results are in accordance with findings that the systemic application of cRGD abrogates accelerated endothelialization and incorporation of EPCs (10), presum-ably by disrupting a haptotactic gradient formed by RGD-containing matrix components.

A major challenge in the present study was to confer the potential benefit of cRGD to a stent system in vivo. The controlled elution of sufficient amounts of hydrophilic peptides has not yet been established. Drug-eluting stents shown to be effective in animal models and clinical trials to date have typically incorporated hydrophobic substances (30,31). A stent coating in our study was designed to enable the elution of 67 µg cRGD over more than 72 h after simulated use and expansion of the stent. The HPLC and liquid chromatography-MS confirmed the presence of cRGD in the tissue surrounding the stent at 4 weeks. Moreover, this was sufficient to successfully attract infused porcine EPCs, as well as endogenous CD34+ cells for effective re-endothelialization. Our results clearly showed that the initial recruitment of infused EPCs from the circulation was markedly increased on cRGD-loaded versus control stents. Taken together with the improved coverage as shown by CD34 staining at later time points (4 and 12 weeks), our data show that loading with cRGD acts to accelerate re-endothelialization by recruiting circulating EPCs. Moreover, the results are in accordance with those from a recent clinical trial using a CD34 antibody-coated stent for effective improvement of endothelialization (32).

Beyond promoting endothelialization to limit neointimal growth, the mobilization of EPCs has been nicely correlated with an increase in segmental NO production after placement of VEGF-2 gene-eluting stents (26), suggesting that the increased EPC recruitment in cRGD-coated stents may be accompanied by a similar improvement of endothelial function.

Finally, the cRGD-loaded polymer stents were tested using a well-established porcine animal model of arterial injury in which the 3-month post-stent follow-up is comparable with 12 to 18 months of follow-up data in humans (33). Results presented here indicate a long-term protective effect of local cRGD delivery on neointimal hyperplasia. Importantly, the polymer showed an excellent biocompatibility in vivo and did not enhance neointimal hyperplasia by itself compared with bare metal stents, and the duration of effect extended beyond that seen in a similar model using sirolimus-eluting stents (21). However, because all data and parameters obtained were collected in an animal model without major spontaneous atherosclerosis, the conclusions drawn might only apply to nondiseased arteries.

The apparent discrepancy in the efficacy of the cRGD stents between 4 and 12 weeks may be explained by the finding that neointimal growth in pigs and humans can continue at later time points amounting to a maximum at 3 months (34), as evident by a difference between 4 and 12 weeks in our control groups. This corresponds to the notion that the time course of re-endothelialization in pig models reported by different groups may substantially vary (6). The use of electron microscopy as a sensitive technology for the detection of (not) endothelialized stent segments enabled us to confirm that re-endothelialization was indeed not com-
plete at four weeks and thus may rather be delayed to later time points in our model. Hence, the benefit of endothelialization in inhibiting neointimal growth (e.g., by limiting SMC recruitment and proliferation) may only be fully accomplished when endothelial recovery has almost been completed at time points later than four weeks. Because endothelialization at four weeks was further improved on cRGD versus control stents in our study, concomitant with the recruitment of EPCs supported by the in vitro results, this may represent the likely mechanism for limited neointimal growth at later time points.

In summary, our data indicate a crucial role of αvβ3-integrins for the homing of EPCs to injured arterial segments after stent implantation. Thus, this class of molecules represents a promising target for drug development to accelerate healing and reduce restenosis after vascular injury.

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REFERENCES


22. Liehn EA, Schober A, Weber C. Blockade of keratinocyte-derived chemokine inhibits endothelial recovery and enhances plaque forma-


28. Deb A, Selding KA, Wang S, Reeder M, Simper D, Caplice NM. Integrin profile and in vivo homing of human smooth muscle progeni-


33. Liehn EA, Schober A, Weber C. Blockade of keratinocyte-derived chemokine inhibits endothelial recovery and enhances plaque forma-


