

EMAP-II downregulation contributes to the beneficial effects of rapamycin after vascular injury

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Aims Neointima formation after vascular injury is strongly associated with inflammation. Rapamycin inhibits human neointima formation and reduces expression of the proinflammatory cytokine endothelial-monocyte activating peptide II (EMAP-II) *in vitro*. Here we investigated the interplay between EMAP-II and rapamycin after vascular injury *in vivo*.

Methods and results In a mouse model of vascular injury, mice were either not treated, given everolimus, a rapamycin derivate, or subjected to simultaneous challenge with everolimus and EMAP-II. EMAP-II expression was measured in coronary artery smooth muscle cells (CASMC) and monocytic cells *in vitro* and in patients after percutaneous coronary intervention (PCI). After vascular injury, rapamycin reduced neointima formation and adventitial thickening. Immunohistochemistry revealed reduced EMAP-II protein expression and suppressed recruitment of inflammatory cells. Simultaneous challenge with EMAP-II counteracted these effects of rapamycin. Expression of EMAP-II and its inhibition by rapamycin was confirmed in CASMC and monocytic cells. In patients, EMAP-II upregulation was confined to PCI of distal coronary artery segments and profoundly suppressed by oral rapamycin treatment.

Conclusion These data suggest important yet unrecognized roles of EMAP-II and adventitial inflammation in neointima formation: Through inhibition of EMAP-II, rapamycin reduces the recruitment of inflammatory cells to the adventitia and supports an early and bland healing.

1. Introduction

Restenosis is a major limitation of percutaneous angioplasty procedures. After stent implantation, it is mainly caused by neointima formation. The recruitment of inflammatory cells correlates with neointima formation in humans.¹ Additionally, apoptosis occurs shortly after vascular injury and is associated with the induction of inflammation.^{2,3} Inhibition of apoptosis, which is a trigger for arterial inflammation, as well as inhibition of the recruitment of inflammatory cells results in decreased neointima formation after balloon angioplasty in rabbits.⁴ In an ischaemia-reperfusion model, apoptosis and subsequent inflammation are linked to endothelial-monocyte activating peptide II (EMAP-II), a proinflammatory cytokine.^{5–7} EMAP-II is activated during apoptosis by cleavage of pro-EMAP through caspases⁸ and

enhances the recruitment of inflammatory cells by its chemotactic properties. Likewise, sites of tissue remodelling in mouse embryo, wherein many apoptotic cells are present, co-localized with EMAP-II mRNA (messenger ribonucleic acid) expression and presence of macrophages.⁸ Besides its chemotactic effect on inflammatory cells, EMAP-II increases the adhesiveness of coronary artery smooth muscle cells (CASMC) for monocytes.⁹ EMAP-II also has anti-angiogenic properties,¹⁰ which are explained by its pro-apoptotic effect on endothelial cells.¹¹ In a human organ culture model, we demonstrated that EMAP-II is upregulated after angioplasty *ex vivo* and downregulated by rapamycin.¹² Furthermore, we showed that rapamycin reduces EMAP-II mRNA expression and that reduction of active EMAP-II levels by caspase inhibitors mimics the functional effect of rapamycin on CASMC resulting in decreased adhesiveness for monocytic cells.⁹

Using drug-eluting stents, neointima formation can be dramatically decreased by local delivery of rapamycin, an anti-proliferative as well as anti-inflammatory drug.¹³

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Although there is elaborate information on the direct effect of rapamycin on cell proliferation, it is poorly understood how rapamycin acts on indirect aspects of neointima formation such as recruitment of inflammatory cells and re-endothelialization after vascular injury.

Here, we investigated the role of EMAP-II and the interplay of rapamycin with EMAP-II in neointima formation *in vivo*, using a mouse model of arterial injury leading to neointima formation.

2. Methods

2.1 Animals

Specific pathogen-free 129S1/SvIMJ mice were obtained from Charles River Laboratories (Sulzfeld, Deutschland). Mice weighing 20–35 g were used for the experiments, kept on a 12 h day/night cycle and fed regular chow. All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.2 Model of restenosis by wire-mediated arterial injury

Surgery was carried out using a microscope (Carl Zeiss, Deutschland) and microsurgical instruments (FST, Heidelberg) as previously described.³ At the time points indicated, mice were euthanized by inhalation of anaesthesia using a whole body chamber and dislocation of the cervical spine. At death, the mice were quickly perfused with ice cold ringer solution and prepped on ice. The femoral artery was dissected free again and 5 mm of the vessel beginning at the insertion of the previously used side branch were excised. Samples were fixed in 6% paraformaldehyde overnight at 4°C, and embedded in paraffin. Cross-sections (2 µm) were stained with Elastica-van-Gieson for morphometric analysis or were used for immunohistochemical analysis.

2.3 Study design

Mice underwent femoral artery injury to induce neointima formation as described.³ Fifteen mice remained untreated after injury and served as a reference of neointima formation and were euthanized 7, 14, and 28 days after vascular injury. Twelve mice received everolimus (Novartis Pharma, Basel, Switzerland), an orally applicable sirolimus derivate. Compared with sirolimus, everolimus absorbs to local tissue more rapidly, possesses longer cellular residence time and activity and has similar properties in reducing restenosis when delivered by drug-eluting-stents.¹⁴ Everolimus was administered orally by sterile gavage feeding cannulae. Preliminary dose-response-studies determined that an initial dose of 10 mg/kg body weight/day for 3 days before injury, followed by a maintenance dose of 5 mg/kg body weight/day until euthanization at day 14 after vascular injury was effective to inhibit neointima formation. All mice receiving everolimus were thus treated according to this regimen. Everolimus was kindly provided by Novartis (Basel, Switzerland).

To investigate the effects of EMAP-II after vascular injury, neointima formation was assessed under influence of intraperitoneally administered EMAP-II. With the EMAP^{-/-} mouse being lethal, rapamycin treatment was used to suppress endogenous EMAP-II production and other inflammatory processes. By simultaneous challenge with EMAP-II and treatment with rapamycin, the unique properties of EMAP-II were further delineated. EMAP-II was given to six mice concomitantly treated with rapamycin. According to an earlier study, which explored the clearance of EMAP-II,¹⁰ a dose of 1 µg was injected every 12 h over 7 days after arterial injury.

2.4 Morphometric and histological analysis

For morphometric analysis, 10 cross-sections from each vessel were used. The first section was adjacent to the side branch used for guide wire insertion, and subsequent sections were obtained at a distance of 66 µm. Digital microscopic images (10×) of the sections taken with AxioVision 2.0 (Carl Zeiss Vision GmbH) were analysed using ScionImagebeta 4.0 software (Scion Corporation). By measuring the circumference of the lumen, the internal elastic membrane (IEL), the external elastic membrane (EEL), and the border of the adventitia in all 10 cross-sections per vessel, the following parameters were calculated: The adventitial area is the area between the vessel border and the EEL, the medial area comprises the space between IEL and EEL, and the neointimal area represents the space between IEL and lumen. Uninjured vessels served as controls ($n = 5$). For determination of re-endothelialization, the relative part of the lumen covered by CD31 positive cells was assessed by the same software. For quantification of neointimal cells, cellular nuclei were counted within the IEL in four subsequent cross-sections per vessel. For quantification of adventitial cells, the number of nuclei was counted in four 10 000 µm² sized adventitial areas called high power field of representative sections and adjusted to the adventitial area.

2.5 Immunohistochemistry

Paraffin sections (2 µm) were deparaffinized, dehydrated and, for antigen retrieval, pressure-cooked for 4 min in citrate buffer (10 mM, pH 6.0), followed by blocking of endogenous peroxidase (3% H₂O₂; 15 min). For EMAP-II staining, a polyclonal antibody (SA2846, Eurogentec, 1:500) and the Vectastain ABC/AEC detection kit were used. For primary antibodies against CD3 (Serotec, 1:400), CD31 (Santa Cruz Biotechnology, 1:200), CD45 (BD Pharmingen, 1:200) and mac-2 (Cedarlane, 1:1000) immunostaining was performed using the 'Dako Autostainer' (DakoCytomation, Hamburg, Germany) and applying the streptavidin-peroxidase technique (Dako ChemMate Detection Kit). For quantification, positive cells were counted as above, three or more sections were analysed per group and the total number of positive adventitial cells was calculated with the mean adventitial area per group.

A fluorescein *in Situ* Cell Death Detection Kit (TUNEL technology, Roche) was used according to the manufacture's specifications. Nuclei were counterstained with DAPI (SigmaRoche).

2.6 Coronary artery smooth muscle cell culture for wounding

CASMC (Clonetics, USA) were cultured according to manufacturer's instruction and used at passage 5–7 by transfer into six well plates until 80% confluent. Cells were serum starved 24 h before treatment. Wounding of confluent monolayer was performed by scratching the surface up and down (seven times in each well) with a 1 mL pipette tip (Eppendorf, Hamburg, Germany). Afterwards, cells were incubated with or without 200 ng/mL rapamycin for a further 24 h. Each condition was done in triplicate; cells were harvested by trypsin-EDTA after 24 h. Total RNA was extracted using the RNeasy kit (Quiagen) and the quantification of RNA was performed by spectrophotometry.

2.7 Determination of relative endothelial-monocyte activating peptide II messenger ribonucleic acid levels in cell culture

Five hundred nanograms total RNA from each sample was used for cDNA synthesis (Superscript III Reverse Transcriptase Kit, Invitrogen, USA). 1/25 of total cDNA was applied for quantitative real time polymerase chain reaction (PCR) (2X Taqman Universal PCR master mix, pre-developed 'assay on demand' Taqman primers/probe for EMAP-II (assay ID Hs00171131_m1), beta-actin (assay ID Hs

99999903_m1, Applied Biosystems, USA) according to manufacturer's recommendation with minor modifications. Standard curves of EMAP-II and beta-actin indicated that PCR amplification efficacy difference of these two genes was negligible. Relative EMAP-II mRNA level was calculated as described previously.¹⁵

2.8 Culture of coronary artery smooth muscle cells and monocytic cells for assessment of endothelial-monocyte activating peptide II protein levels

CASMC (Clonetics) were plated on 24 well plates at a density of 50 000 cells/well. Monomac6 cells which represent human monocytic cells with a closely related pattern of surface receptors and monocyte-like behaviour (Ziegler-Heitbrock *et al.*,¹⁶ kindly provided by R. Schmidt, Technical University, Munich) were seeded in 24 well plates at the same density (50 000 cells/well). All cells were kept in serum-free medium 24 h with or without everolimus (100 ng/mL) before subsequent stimulation with Thrombin (3 U/mL) for 24 h. Afterwards, all cells were directly lysed with 150 μ L lysis buffer (Lämmli-buffer, 50 mM TRIS, 2%SDS and Proteinase Inhibitor Cocktail (Roche)). Before analysis of EMAP-II concentration using an EMAP-II ELISA kit (Biosource), all lysates were concentrated with Microcon YM-10 centrifugal filter units (Millipore) over 20 min at 13 200 r.p.m.

2.9 Assessment of endothelial-monocyte activating peptide II protein levels in patients

For assessment of EMAP-II protein, samples were collected from 33 patients without known haematologic disorders or malignancies undergoing elective percutaneous coronary intervention (PCI) immediately before PCI and 6–8 h after intervention of native coronary arteries. EMAP-II protein concentration was measured using the ChemiKine™ human EMAP-II EIA kit (Chemicon Intl, Temecula, CA, USA) according to the manufacturers protocol.

All patients received acetylsalicylic acid, clopidogrel and heparin or bivalirudin prior to the procedure. *Table 1* outlines the clinical, angiographic, and procedural characteristics of the patients. For

classification of coronary artery segments, the AHA classification from 1975 was used.¹⁷ The coronary segments 1 and 2; and 5, 6, and 11 were defined as proximal, the rest considered as distal.

To evaluate the effects of rapamycin on EMAP-II expression in humans, samples available from the Oral Sirolimus to Inhibit Recurrent In-stent Stenosis (OSIRIS) trial¹⁸ were used. In this trial, patients presenting with in-stent restenosis who underwent PCI were divided into three groups: placebo or usual-dose or high-dose rapamycin started prior to PCI. Compared with the two other groups, the high-dose rapamycin group showed a significantly reduced rate of recurrent restenosis at 6-month angiography. Interestingly, the restenosis rate strongly correlated negatively with the rapamycin concentration at the day of the intervention. Therefore, we compared EMAP-II blood concentrations from the high-dose rapamycin group ($n = 47$) with the placebo group ($n = 41$) with the same EIA kit. After collecting a baseline value at least 1 day prior to PCI, samples were taken on the day of PCI and in time ranges between 1 and 4 days as well as 5–25 days after PCI.

All blood collections were carried out conforming to the principles outlined in the Declaration of Helsinki and was approved by the institutional ethics committee.

2.10 Statistical analysis

All values are presented as mean \pm standard error of mean or median with inter quartile range (IQR from the 25th to 75th percentile) if appropriate. Differences of quantitative data between more than two groups were tested using Kruskal–Wallis and Mann–Whitney U-test for pairwise comparisons. To quantify bivariate correlation between measurement data, Spearman correlation coefficient was calculated. Assessment of EMAP-II expression after PCI of native coronary arteries was complemented by a multivariate analysis to assess for possible influences secondary to differences in baseline variables. After log-transformation to control for non-normal distribution of the target variables, a stepwise linear regression analysis was performed. All tests are two-sided and were performed at a 5% level of significance by using SPSS 10.0 (SPSS Inc., Chicago, IL, USA).

Table 1 Clinical, angiographic and procedural characteristics of the patients

	Only proximal (eight patients)	Distal + proximal (13 patients)	Only distal (12 patients)	<i>P</i> -value
Clinical characteristics				
Age (year)	63.8 \pm 16.5	65.3 \pm 7.4	69.5 \pm 10.2	0.479
Women (%)	0	31	17	0.219
Diabetes (%)	38	23	33	0.770
Hypercholesterolemia (%)	88	100	100	0.214
Arterial hypertension (%)	100	100	100	n/a
Previous myocardial infarction (%)	50	69	17	0.027
Angiographic characteristics				
Left ventricular ejection fraction (%)	54.8 \pm 10.0	56.1 \pm 10.1	58.7 \pm 7.0	0.614
Target vessel				
LCA (%)	63	23	0	0.003
LAD (%)	75	92	50	0.873
LCx (%)	50	23	25	0.174
RCA (%)	13	23	42	0.296
Procedural data				
Number of stents implanted	1.8 \pm 0.7	3.0 \pm 1.2	1.6 \pm 1.0	0.003
Dilations/intervened segment	5.2 \pm 3.7	3.3 \pm 1.9	3.3 \pm 1.7	0.172
Drug-eluting stent (%)	100	100	89	0.430
Paclitaxel (%)	50	8	0	0.006
Sirolimus (%)	50	74	88	0.015
Zotarolimus (%)	0	18	12	0.121

LCA, left main coronary artery; LAD, left anterior descending coronary artery; LCx, left circumflex coronary artery; and RCA, right coronary artery.

3. Results

3.1 Endothelial-monocyte activating peptide II expression was increased during neointima formation in a mouse model *in vivo* and was inhibited by rapamycin

EMAP-II expression was investigated at different time points after wire-injury *in vivo* with respect to rapamycin treatment. Neointima formation occurred after 7 days (data not shown) with further increase 14 days (Figure 1C) and 28 days (Figure 1E) after vascular injury. Compared with uninjured control (Figure 1A and B), EMAP-II expression was present at all time points in subendothelial leukocytes and medial smooth muscle cells (SMCs) (Figure 1D and F). Rapamycin treatment led to reduction of EMAP-II expression (Figure 1H) and to a significant decrease in neointima formation (Figure 1G, quantified in Figure 2A).

3.2 Restoration of endothelial-monocyte activating peptide II counteracted effects of rapamycin after vascular injury in mice

Morphometric analysis was performed in control vessels and 2 weeks after wire injury in untreated, rapamycin-treated, and simultaneously treated mice. Two weeks after arterial injury, exuberant adventitial proliferation, a small increase

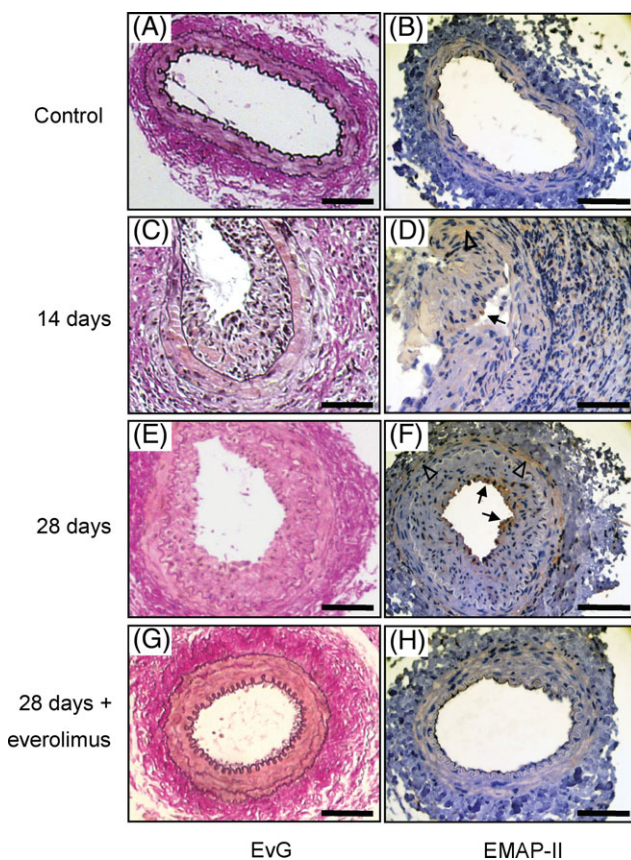


Figure 1 Endothelial-monocyte activating peptide II (EMAP-II) expression in neointima formation and reduction by rapamycin. Elastic-van-Gieson (A, C, E, G) and immunohistochemical staining against EMAP-II (B, D, F, H) of control (A, B) and wire-injured murine *A. femorales* (C–H). EMAP-II is absent in the uninjured control vessel (B), but is detected in subendothelial leukocytes (arrows) and medial smooth muscle cells (arrowheads) 14 days after injury (D) and 28 days after injury (F). Treatment with rapamycin for 28 days led to near abrogation of EMAP-II expression (H). Scale bars indicate 100 μ m.

in medial area and significant neointima formation occurred compared with the uninjured controls (Figure 2A). As expected from previous studies, treatment with rapamycin significantly reduced neointima formation and prevented medial proliferation. Rapamycin also markedly inhibited adventitial thickening (Figure 2A, white bars). Simultaneous administration of EMAP-II in rapamycin-treated mice counteracted the effect of rapamycin and led to a significant increase of neointimal volume compared with mice treated with rapamycin alone, whereas its effect on adventitial thickening was less pronounced (Figure 2A, grey bars).

Furthermore, the cell content of neointima and adventitia was analysed in histological sections. Compared with injured arteries, rapamycin treatment led to markedly reduced cell accumulation in neointima (Figure 2B, lower panel) and adventitia (Figure 2B, upper panel). Measuring this parameter of vascular inflammation, EMAP-II challenge after wire injury increased the cellular content significantly in both compartments (Figure 2B) and counteracted the positive effect of rapamycin.

3.3 Adventitial cell content outweighed neointimal cell content and correlated with neointima formation after vascular injury

Adventitial cell counts exceeded those in the neointima about four to seven-fold (1225 ± 143 , 297 ± 47 and 481 ± 55 vs. 164 ± 11 , 56 ± 5 and 117 ± 25 per section in control, rapamycin- and simultaneously-treated groups). Similar to neointimal cell content¹ (Figure 2B, lower panel), the amount of adventitial cells correlated with the extent of neointima formation (Figure 2B, upper panel).

3.4 Endothelial-monocyte activating peptide II increased recruitment of macrophages, enhanced apoptosis, and decreased re-endothelialization after vascular injury *in vivo*

To further characterize the largely increased adventitial cell content induced by restoration of EMAP-II (Figure 2B), we performed immunohistochemical staining with antibodies against CD3, CD45, mac-2, and c-kit (Figure 3) and quantified the amount of positive cells in the adventitia. The vast majority of the adventitial cells were found to be of leukocytic origin by positive staining for the transleukocyte marker CD45. Staining against mac-2 identified most of them to be macrophages; whereas, only a few CD3 positive T-cells were found 14 days after vascular injury. As described previously for neointimal cells, we found in our model that a distinct percentage of adventitial cells were positive for the haematopoietic progenitor cell marker c-kit after vascular injury, which was reduced significantly by rapamycin treatment (Figure 3). Moreover, rapamycin treatment led to a marked reduction in the recruitment of CD45 and mac-2 positive inflammatory cells to the adventitia. Whereas restoration of EMAP-II did not alter the amount of CD3 or c-kit positive cells in the vessels significantly, it increased the amount of macrophages as demonstrated by CD45 and mac-2 staining (Figure 3).

To assess for possible pro-apoptotic effects of EMAP-II in vascular repair, TUNEL-staining was performed. Consistent with previous data,³ significant apoptosis, mainly confined to the medial SMCs, was observed in our model of vascular injury. Rapamycin treatment led to a marked decrease of

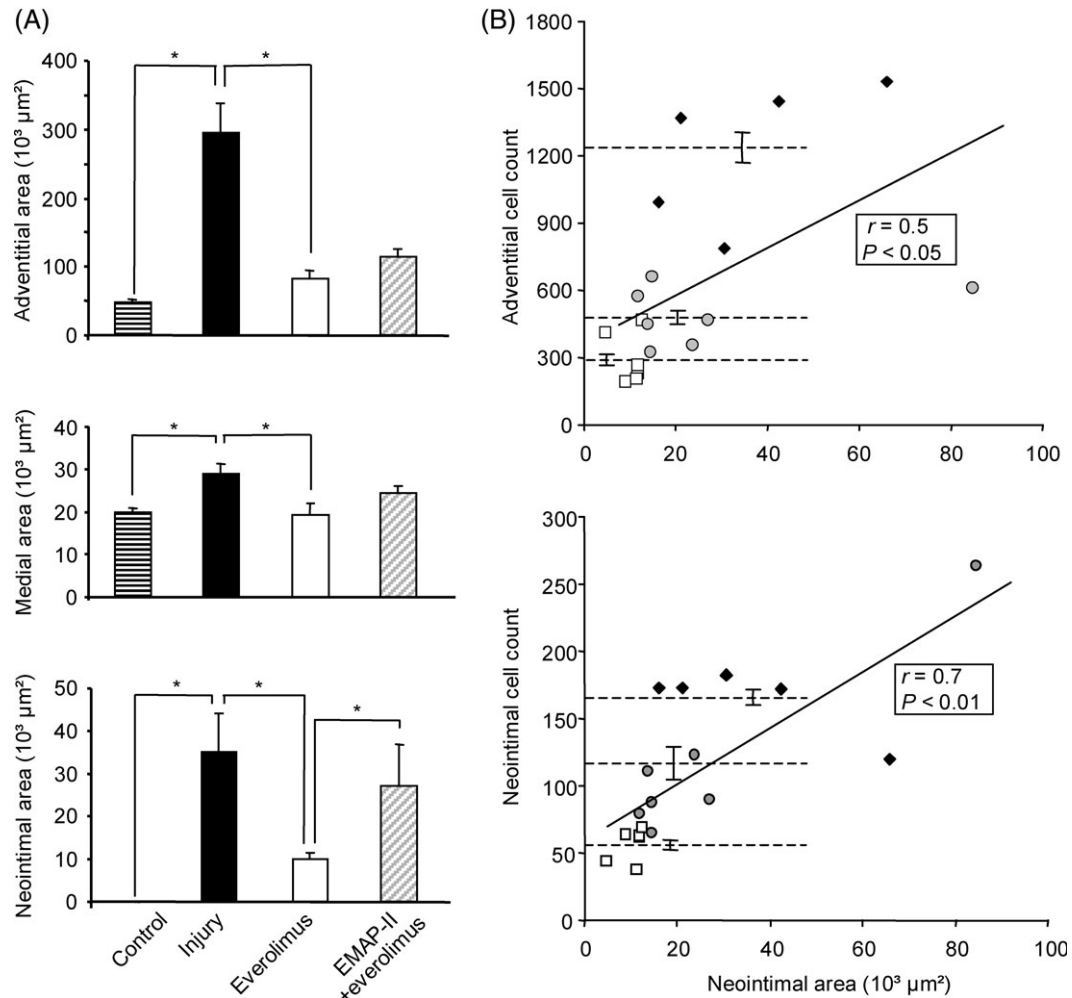


Figure 2 Effects of rapamycin treatment and concomitant endothelial-monocyte activating peptide II (EMAP-II) challenge 14 days after vessel injury. Morphometric analysis of important vessel parameters (A): rapamycin (white columns) limits the increase in adventitial and medial area and significantly inhibits neointima formation. EMAP-II (grey columns) antagonizes the beneficial effects of rapamycin on neointimal area significantly while minor changes were observed regarding the adventitial and medial area. Ten subsequent sections of each vessel were analysed morphometrically 14 days after intervention. (B) Quantification of neointimal and adventitial cells: neointima formation correlates with adventitial ($r = 0.5$, $P < 0.05$; upper panel) and neointimal ($r = 0.7$, $P < 0.01$; lower panel) cell count. EMAP-II challenge also counteracts the reduced cell recruitment achieved by rapamycin treatment in adventitia and neointima (horizontal lines, both $P < 0.05$). Values of untreated mice are represented in black diamonds, of rapamycin treated mice in white boxes and of mice undergoing combined treatment in grey circles; mean values indicated by horizontal lines \pm SEM.

TUNEL⁺ cells in the media. However, concomitant treatment with EMAP-II significantly increased the amount of TUNEL⁺ cells compared with sole rapamycin administration (Figure 4).

Immunohistochemical staining against CD31 was used to quantify the amount of re-endothelialization (Figure 5). On an average $53 (\pm 14)\%$ of the lumen were covered with endothelial cells 2 weeks after vascular injury. Treatment with rapamycin evoked more consistent re-endothelialization ($84 \pm 7\%$, $P = 0.038$) of the injured arteries. Restoration of EMAP-II, caused a significant decrease in re-endothelialization ($42 \pm 12\%$, $P = 0.005$) compared with rapamycin treatment alone.

3.5 Endothelial-monocyte activating peptide II expression was increased in coronary artery smooth muscle cells after mechanical stress

Little is known about molecular mechanisms regulating EMAP-II mRNA expression. In order to mechanistically confirm increased EMAP-II expression after arterial injury,

we tested EMAP-II expression in response to wounding and its modulation by rapamycin *in vitro*. Wounding of CASC increased EMAP-II mRNA expression significantly; whereas, the presence of rapamycin significantly reduced this effect to baseline levels of the untreated control cells. Rapamycin treatment alone reduced the basal levels of EMAP-II mRNA in untreated control cells by about 20% (Figure 6A).

3.6 Endothelial-monocyte activating peptide II expression in coronary artery smooth muscle cells and monocyte cells was reduced by rapamycin

Next, we assessed EMAP-II expression in CASCs and monocyte cells. At baseline, CASC showed a 1.4-fold higher EMAP-II expression than MonoMac6 cells. After stimulation with thrombin, both cell lines showed a significant increase in EMAP-II expression which could be abrogated by treatment with rapamycin. Under all conditions, EMAP-II expression in CASCs remained higher than in MonoMac6 cells (Figure 6B).

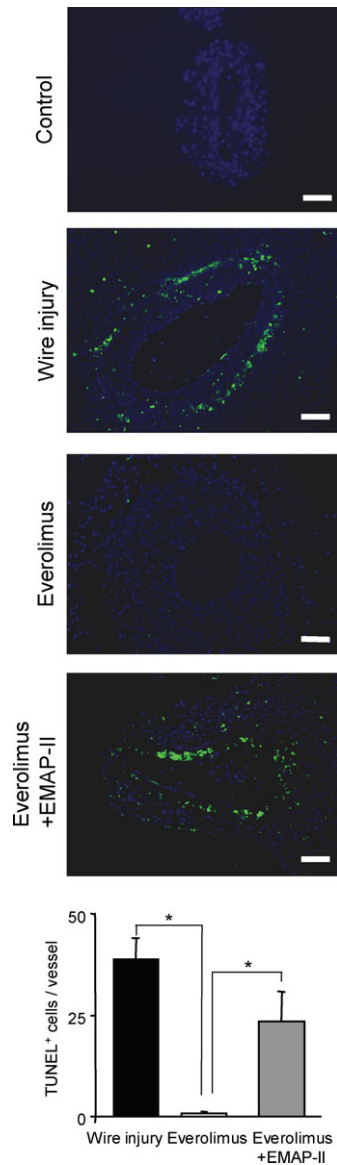


Figure 4 Apoptosis after vessel injury and the effects of rapamycin and endothelial-monocyte activating peptide II (EMAP-II). Apoptotic medial smooth muscle cells assessed by TUNEL-staining are evident after vascular injury but not with rapamycin treatment. Concomitant EMAP-II challenge increases the amount of TUNEL⁺ cells. * $P < 0.05$. Scale bars indicate 100 μm .

4. Discussion

Rapamycin reduces the risk of in-stent restenosis by inhibiting neointimal proliferation in patients.¹³ However, the underlying mechanisms of how rapamycin governs neointima formation are not yet understood in detail. Here, we systematically investigated the role of EMAP-II and the interplay of rapamycin with EMAP-II *in vivo* in a mouse model of neointima formation and in patients.

We show for the first time: (i) EMAP-II is upregulated during neointima formation *in vivo*; (ii) the adventitial inflammatory reaction is positively correlated to the extent of neointima formation and is significantly reduced by rapamycin; (iii) rapamycin inhibits upregulation of EMAP-II and counteracts the pro-inflammatory effects of EMAP-II. (iv) likewise, reduced recruitment of macrophages

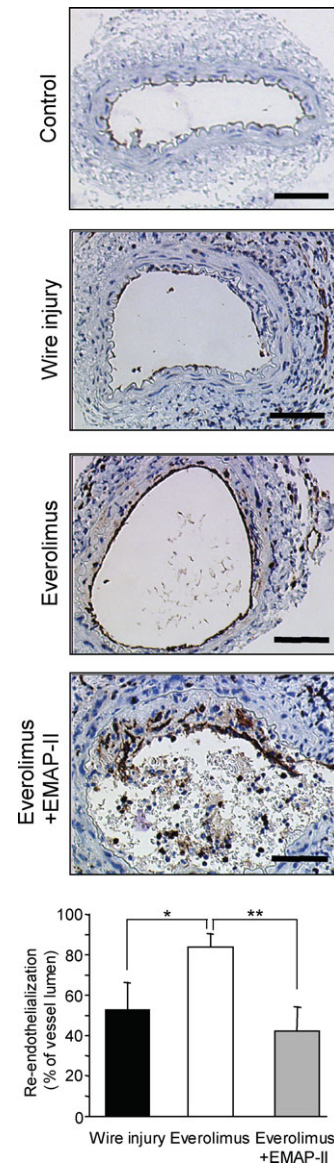


Figure 5 Effects of rapamycin and endothelial-monocyte activating peptide II (EMAP-II) on re-endothelialization after vessel injury. Endothelial cells positive for CD31 are found in untreated, rapamycin treated and in vessels with combined treatment. Quantification of endothelialization revealed a beneficial effect of rapamycin, which was abrogated by concomitant EMAP-II challenge. (* $P < 0.05$, ** $P < 0.01$). Scale bars indicate 100 μm .

to the site of vascular injury by rapamycin is counteracted by EMAP-II challenge; (v) rapamycin improves re-endothelialization, which is abrogated by EMAP-II, and (vi) EMAP-II expression is induced after mechanical injury in human CASCs and patients, and downregulated by rapamycin treatment.

This study reveals that EMAP-II plays an important role in the inflammatory response to vascular injury *in vivo*. In congruence with prior *ex-vivo* and *in vitro* data, we confirmed increased EMAP-II protein expression during neointima formation after vascular injury¹² and its inhibition by rapamycin⁹ *in vivo*. The observed expression in subendothelial leukocytes and medial SMCs is in line with its known expression in stimulated monocytes and vascular SMCs.²³ Since the model of injury used in our study leads to induction of medial cell apoptosis,³ the observed upregulation

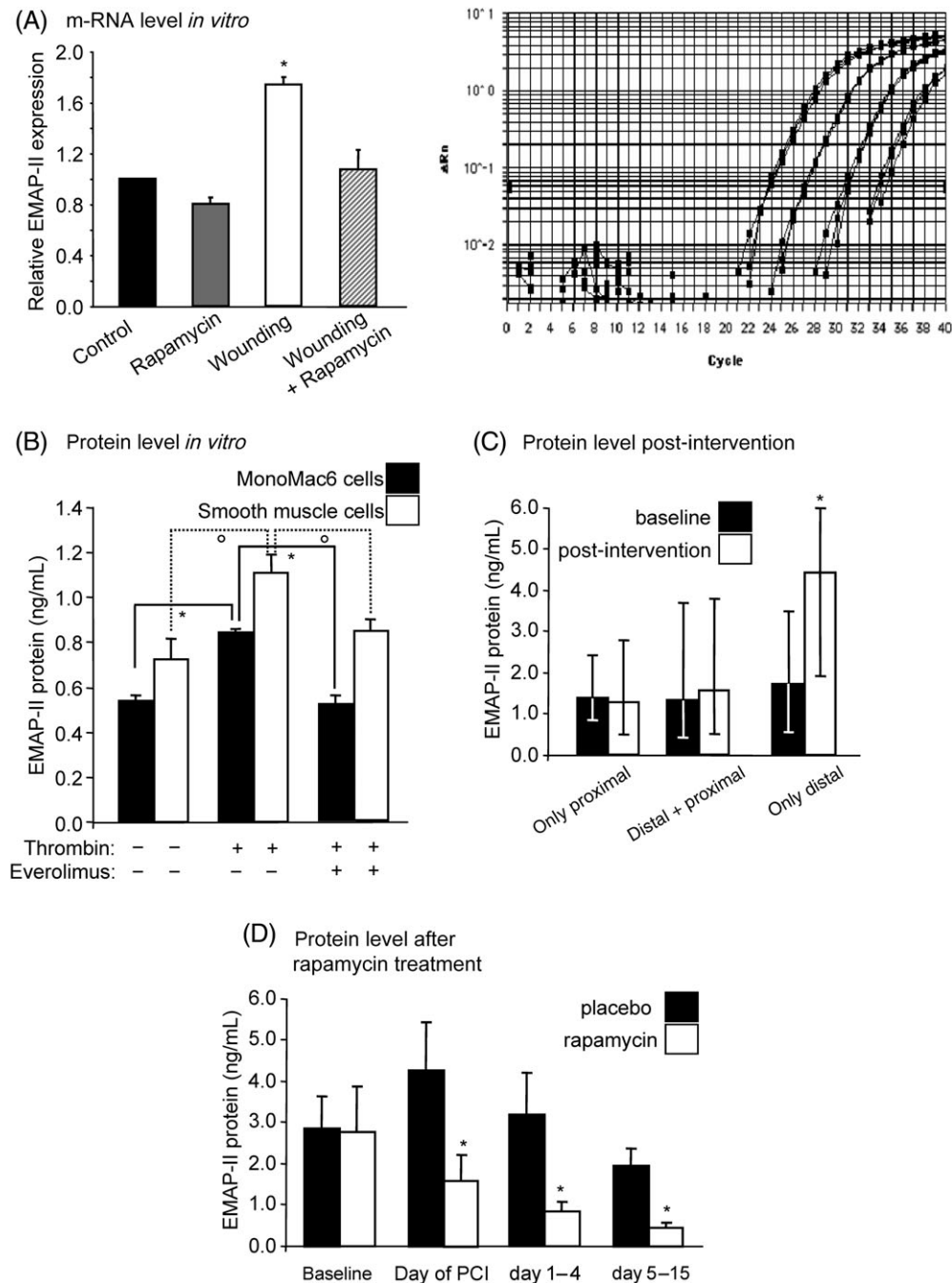


Figure 6 Regulation of endothelial-monocyte activating peptide II (EMAP-II) expression by mechanical stress and rapamycin. (A) EMAP-II-mRNA levels in human coronary artery smooth muscle cells (CASMC) in response to wounding and effect of rapamycin. On the left, relative EMAP-II mRNA levels from CASMC 24 h after treatment with/without wounding and with/without 200 ng/mL rapamycin are shown. On the right, the amplification curves of EMAP-II in the real-time polymerase chain reaction (PCR) are displayed. Wounding increased EMAP-II mRNA (messenger ribonucleic acid), which was reduced after treatment with rapamycin to about control levels ($*P < 0.05$). (B) EMAP-II protein levels in human CASMC and MonoMac6 cells in response to thrombin stimulation and effect of rapamycin. Increased EMAP-II expression by thrombin was inhibited by rapamycin in both cell types ($*P < 0.01$, $^{\circ}P < 0.05$). (C) Assessment of EMAP-II protein levels 6–8 h after percutaneous coronary intervention shows that upregulation is limited to patients with intervention in distal coronary artery segments (median \pm IQR (inter quartile range), $*P < 0.01$). (D) EMAP-II protein levels in patients from the Oral Sirolimus to Inhibit Recurrent In-stent Stenosis (OSIRIS)-trial show marked suppression under treatment with oral rapamycin (mean \pm SEM, $*P < 0.01$).

of EMAP-II is consistent with its known expression in tissues with high rates of apoptosis and inflammation.^{5,8}

Furthermore, we could show marked suppression of apoptosis by rapamycin after vascular injury, whereas restoration of EMAP-II leads to increased apoptosis of medial SMCs. Since EMAP-II does not have pro-apoptotic effects on mesenchymal cells¹¹ *in vitro*, our data suggest that EMAP-II increases recruitment of inflammatory cells such as

monocytes and neutrophils and thereby promotes a pro-apoptotic environment.

The occurrence of restenosis is associated with a high inflammatory cell density of neointima. Farb *et al.*¹ demonstrated that the neointimal inflammatory cell content was 2.4-fold greater in stents with restenosis vs. in stents with no restenosis. In our study, we demonstrate that inflammatory cell accumulation in the adventitia outweighs by far

the neointimal cell content and significantly correlates with neointima formation. These findings underline the hypothesis that the adventitia is the major site of inflammatory changes after vascular injury.²⁴ Suppressing EMAP-II expression, rapamycin reduced the inflammatory cell content in the adventitia and neointima after vascular injury significantly and concordantly decreased neointima formation *in vivo*. In line with our data, it was recently shown that adventitial delivery of rapamycin reduces neointima formation,²⁵ further emphasizing that the adventitial inflammatory reaction plays a pivotal role in the pathogenesis of neointima formation. This is not in contrast with the notion that adventitial cells are not a significant contributor to neointimal cell content since the adventitial inflammatory response may trigger enhanced recruitment of circulating progenitor cells.²⁴

In human vascular repair, monocytes and neutrophils play a crucial role.²⁶ EMAP-II is a potent cytokine in this context since it induces migration of monocytes and chemotaxis of neutrophils.⁶ Likewise, EMAP-II reversed the rapamycin-associated inhibition of monocyte recruitment and counteracted as well the beneficial effect of rapamycin on neointima formation. Active EMAP-II induced an inflammatory reaction not only at the endovascular site of the injury but also aggravated the inflammatory response of the adventitial tissue, thereby contributing to neointima formation after vascular injury. In addition, our study shows that the beneficial effect of rapamycin after vascular injury is at least partly mediated by suppression of active EMAP-II. As proposed in an earlier study,¹² we confirmed that rapamycin dramatically reduces the recruitment of inflammatory and haematopoietic cells to the vessel wall *in vivo*.

Moreover, we found that rapamycin treatment significantly facilitated re-endothelialization. This is in line with its suppressive effect on EMAP-II considering that EMAP-II has pro-apoptotic effects on endothelial cells.^{10,11} Likewise, we reproduced impaired re-endothelialization by addition of EMAP-II to rapamycin treatment.

This supports the notion that the beneficial effects of rapamycin on re-endothelialization are secondary to suppression of active EMAP-II. On first glance, our findings conflict with recent data where decreased re-endothelialization was observed after local delivery of rapamycin.²⁵ However, in this study, extraluminal local drug administration suggests high tissue drug concentrations probably even exceeding previously reported tissue levels.²⁷ This concurs with results of Farb *et al.*²⁸ who reported incomplete neointimal healing of stented areas after high dose oral everolimus treatment. Furthermore, a high rate of re-endothelialization was observed with low dose everolimus treatment in the same study.²⁸ Thus, the effect of rapamycin on re-endothelialization appears to be dose dependent. Ultimately, we cannot exclude differences between everolimus and sirolimus in this aspect.

Together, our data may be crucial for our understanding how rapamycin prevents restenosis. By inhibition of apoptosis, it prevents early inflammatory processes mainly located in the adventitia, supports a bland wound healing, and early re-endothelialization. Thereby rapamycin helps to avoid chronic inflammation which is associated with increased restenosis.¹ However, its beneficial effects in the clinical setting appear to be obscured by its delivery through polymer-coated stents which enhance arterial inflammation

and delay arterial healing resulting in delayed re-endothelialization.^{29–31}

To further prove the upregulation of EMAP-II after mechanical injury in humans, we assessed its expression in CASMCs patients. *In vitro*, EMAP-II mRNA was upregulated by mechanical stress in CASMCs which was prevented by rapamycin. On the protein level, congruent effects were seen in CASMCs as well as monocytic cells after stimulation with thrombin. Using this way of stimulation, we were able to compare EMAP-II expression in CASMCs and monocytic cells under similar conditions. Interestingly, CASMCs showed on an average a 1.4-fold higher EMAP-II expression pointing towards an important role as immune-modulating cells in the recruitment of circulating macrophages and neutrophils after vascular injury.

At last, we found a significant upregulation of EMAP-II protein in patients after PCI. Interestingly, the upregulation was confined to mechanical injury of smaller vessels, which are known to have higher rates of restenosis when treated with bare metal stents.¹⁹ However, our data is limited by the fact that our patients almost exclusively received drug-eluting stents. Thus a potential influence of the stent type on EMAP-II expression cannot be ruled out, though we did not find an influence of the sirolimus-eluting stents or number of implanted stents on EMAP-II expression in the multivariate analysis.

At this time, it remains therefore speculative if this observation is due to biological differences of the coronary artery segments and their plaque composition or if the relative vessel injury is higher in the smaller vessels.

Yet these data demonstrate that EMAP-II is also regulated in patients and provide a rationale to further explore its role in human vascular repair. Furthermore, oral rapamycin decreased blood levels of EMAP-II protein in patients conferring the inhibitory effect of rapamycin on EMAP-II and vascular inflammation to the clinical situation in patients.

In conclusion, we provide strong evidence for the hypothesis that EMAP-II is an important mediator of inflammation after vascular injury and that the beneficial effects of rapamycin on vascular inflammation are mediated by downregulating this cytokine.

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References

1. Farb A, Weber DK, Kolodgie FD, Burke AP, Virmani R. Morphological predictors of restenosis after coronary stenting in humans. *Circulation* 2002; 105:2974–2980.
2. Roque M, Fallon JT, Badimon JJ, Zhang WX, Taubman MB, Reis ED. Mouse model of femoral artery denudation injury associated with the rapid accumulation of adhesion molecules on the luminal surface and recruitment of neutrophils. *Arterioscler Thromb Vasc Biol* 2000;20:335–342.
3. Sata M, Maejima Y, Adachi F, Fukino K, Saiura A, Sugiura S *et al.* A mouse model of vascular injury that induces rapid onset of medial cell apoptosis followed by reproducible neointimal hyperplasia. *J Mol Cell Cardiol* 2000; 32:2097–2104.

4. Beohar N, Flaherty JD, Davidson CJ, Maynard RC, Robbins JD, Shah AP *et al*. Antirestenotic effects of a locally delivered caspase inhibitor in a balloon injury model. *Circulation* 2004;**109**:108–113.
5. Daemen MA, van' V, Denecker G, Heemskerk VH, Wolfs TG, Clauss M *et al*. Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation. *J Clin Invest* 1999;**104**:541–549.
6. Kao J, Ryan J, Brett G, Chen J, Shen H, Fan YG *et al*. Endothelial monocyte-activating polypeptide II. A novel tumor-derived polypeptide that activates host-response mechanisms. *J Biol Chem* 1992;**267**:20239–20247.
7. Kao J, Fan YG, Haehnel I, Brett J, Greenberg S, Clauss M *et al*. A peptide derived from the amino terminus of endothelial-monocyte-activating polypeptide II modulates mononuclear and polymorphonuclear leukocyte functions, defines an apparently novel cellular interaction site, and induces an acute inflammatory response. *J Biol Chem* 1994;**269**:9774–9782.
8. Knies UE, Behrendorf HA, Mitchell CA, Deutsch U, Risau W, Drexler HC *et al*. Regulation of endothelial monocyte-activating polypeptide II release by apoptosis. *Proc Natl Acad Sci USA* 1998;**95**:12322–12327.
9. Zohnhöfer D, Nührenberg TG, Neumann FJ, Richter T, May AE, Schmidt R *et al*. Rapamycin effects transcriptional programs in smooth muscle cells controlling proliferative and inflammatory properties. *Mol Pharmacol* 2004;**65**:880–889.
10. Schwarz MA, Kandel J, Brett J, Li J, Hayward J, Schwarz RE *et al*. Endothelial-monocyte activating polypeptide II, a novel antitumor cytokine that suppresses primary and metastatic tumor growth and induces apoptosis in growing endothelial cells. *J Exp Med* 1999;**190**:341–354.
11. Berger AC, Alexander HR, Tang G, Wu PS, Hewitt SM, Turner E *et al*. Endothelial monocyte activating polypeptide II induces endothelial cell apoptosis and may inhibit tumor angiogenesis. *Microvasc Res* 2000;**60**:70–80.
12. Nührenberg TG, Voisard R, Fahlisch F, Rudelius M, Braun J, Gschwend J *et al*. Rapamycin attenuates vascular wall inflammation and progenitor cell promoters after angioplasty. *FASEB J* 2005;**19**:246–248.
13. Moses JW, Leon MB, Popma JJ, Fitzgerald PJ, Holmes DR, O'Shaughnessy C *et al*. Sirolimus-eluting stents versus standard stents in patients with stenosis in a native coronary artery. *N Engl J Med* 2003;**349**:1315–1323.
14. Costa RA, Lansky AJ, Mintz GS, Mehran R, Tsuchiya Y, Negoita M *et al*. Angiographic results of the first human experience with everolimus-eluting stents for the treatment of coronary lesions (the FUTURE I trial). *Am J Cardiol* 2005;**95**:113–116.
15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;**25**:402–408.
16. Ziegler-Heitbrock HW, Thiel E, Futterer A, Herzog V, Wirtz A, Riethmüller G. Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. *Int J Cancer* 1988;**41**:456–461.
17. Austen WG, Edwards JE, Frye RL, Gensini GG, Gott VL, Griffith LS *et al*. A reporting system on patients evaluated for coronary artery disease. Report of the Ad Hoc Committee for Grading of Coronary Artery Disease, Council on Cardiovascular Surgery, American Heart Association. *Circulation* 1975;**51**:5–40.
18. Hausleiter J, Kastrati A, Mehilli J, Vogeser M, Zohnhöfer D, Schühlen H *et al*. Randomized, double-blind, placebo-controlled trial of oral sirolimus for restenosis prevention in patients with in-stent restenosis: the Oral Sirolimus to Inhibit Recurrent In-stent Stenosis (OSIRIS) trial. *Circulation* 2004;**110**:790–795.
19. Elezi S, Kastrati A, Neumann FJ, Hadamitzky M, Dirschinger J, Schomig A. Vessel size and long-term outcome after coronary stent placement. *Circulation* 1998;**98**:1875–1880.
20. Schampaert E, Cohen EA, Schluter M, Reeves F, Traboulsi M, Title LM *et al*. The Canadian study of the sirolimus-eluting stent in the treatment of patients with long de novo lesions in small native coronary arteries (C-SIRIUS). *J Am Coll Cardiol* 2004;**43**:1110–1115.
21. Elezi S, Dibra A, Mehilli J, Pache J, Wessely R, Schömig A *et al*. Vessel size and outcome after coronary drug-eluting stent placement: results from a large cohort of patients treated with sirolimus- or paclitaxel-eluting stents. *J Am Coll Cardiol* 2006;**48**:1304–1309.
22. Schofer J, Schluter M, Gershlick AH, Wijns W, Garcia E, Schampaert E *et al*. Sirolimus-eluting stents for treatment of patients with long atherosclerotic lesions in small coronary arteries: double-blind, randomised controlled trial (E-SIRIUS). *Lancet* 2003;**362**:1093–1099.
23. Shane JW, Janardhan KS, Singh B. Expression and function of endothelial monocyte-activating polypeptide-II in acute lung inflammation. *Inflamm Res* 2007;**56**:175–181.
24. Wilcox JN, Okamoto EI, Nakahara KI, Vinten-Johansen J. Perivascular responses after angioplasty which may contribute to postangioplasty restenosis: a role for circulating myofibroblast precursors? *Ann NY Acad Sci* 2001;**947**:68–90.
25. Fukuda D, Sata M, Tanaka K, Nagai R. Potent inhibitory effect of sirolimus on circulating vascular progenitor cells. *Circulation* 2005;**111**:926–931.
26. Komatsu R, Ueda M, Naruko T, Kojima A, Becker AE. Neointimal tissue response at sites of coronary stenting in humans: macroscopic, histological, and immunohistochemical analyses. *Circulation* 1998;**98**:224–233.
27. Suzuki T, Kopia G, Hayashi S, Bailey LR, Llanos G, Wilensky R *et al*. Stent-based delivery of sirolimus reduces neointimal formation in a porcine coronary model. *Circulation* 2001;**104**:1188–1193.
28. Farb A, John M, Acampado E, Kolodgie FD, Prescott MF, Virmani R. Oral everolimus inhibits in-stent neointimal growth. *Circulation* 2002;**106**:2379–2384.
29. Virmani R, Farb A, Guagliumi G, Kolodgie FD. Drug-eluting stents: caution and concerns for long-term outcome. *Coron Artery Dis* 2004;**15**:313–318.
30. Kotani J, Awata M, Nanto S, Uematsu M, Oshima F, Minamiguchi H *et al*. Incomplete neointimal coverage of sirolimus-eluting stents: angioscopic findings. *J Am Coll Cardiol* 2006;**47**:2108–2111.
31. Finn AV, Kolodgie FD, Harnek J, Guerrero LJ, Acampado E, Tefera K *et al*. Differential response of delayed healing and persistent inflammation at sites of overlapping sirolimus- or paclitaxel-eluting stents. *Circulation* 2005;**112**:270–278.