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.

KEYWORDS Restenosis; Inflammation; Adventitia; Endothelium; Drugs

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 Additiona-ly, 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 caspas-8 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) 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 rapa-mycin 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.

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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. 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 a 3 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 (2μm) were stained with Elastica-van Gieson for morphometric analysis or used for immunohistochemical analysis.

2.3 Study design

Mice underwent femoral artery injury to induce neointima formation as described. 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. 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, 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 (2μm) 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 Phar-mingen, 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 manufacturer’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 RNAeasy kit (Qiagen) 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 mononcytic cells for assessment of endothelial-mononcytic 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 monocyctic cells with a closely related pattern of surface receptors and monocyte-like behaviour (Ziegler-Heitbrock et al., 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 ml 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-mononcytic 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 ChemiKineTM human EMAP-II EIA kit (Chemicon Itl, 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 other two 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 ± 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

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Only proximal (eight patients)</th>
<th>Distal ± proximal (13 patients)</th>
<th>Only distal (12 patients)</th>
<th>P-value</th>
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</thead>
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<tr>
<td>Age (year)</td>
<td>63.8 ± 16.5</td>
<td>65.3 ± 7.4</td>
<td>69.5 ± 10.2</td>
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<td>Women (%)</td>
<td>0</td>
<td>31</td>
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<td>Diabetes (%)</td>
<td>38</td>
<td>23</td>
<td>33</td>
<td>0.770</td>
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<td>Hypercholesterolemia (%)</td>
<td>88</td>
<td>100</td>
<td>100</td>
<td>0.214</td>
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<tr>
<td>Arterial hypertension (%)</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Previous myocardial infarction (%)</td>
<td>50</td>
<td>69</td>
<td>17</td>
<td>0.027</td>
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<tr>
<td>Angiographic characteristics</td>
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<td></td>
<td></td>
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<tr>
<td>LCA (%)</td>
<td>63</td>
<td>23</td>
<td>0</td>
<td>0.003</td>
</tr>
<tr>
<td>LAD (%)</td>
<td>75</td>
<td>92</td>
<td>50</td>
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</tr>
<tr>
<td>LCx (%)</td>
<td>50</td>
<td>23</td>
<td>25</td>
<td>0.174</td>
</tr>
<tr>
<td>RCA (%)</td>
<td>13</td>
<td>23</td>
<td>42</td>
<td>0.296</td>
</tr>
<tr>
<td>Procedural data</td>
<td></td>
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<td></td>
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<tr>
<td>Number of stents implanted</td>
<td>1.8 ± 0.7</td>
<td>3.0 ± 1.2</td>
<td>1.6 ± 1.0</td>
<td>0.003</td>
</tr>
<tr>
<td>Dilations/intervened segment</td>
<td>5.2 ± 3.7</td>
<td>3.3 ± 1.9</td>
<td>3.3 ± 1.7</td>
<td>0.172</td>
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<tr>
<td>Drug-eluting stent (%)</td>
<td>100</td>
<td>100</td>
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<td>Paclitaxel (%)</td>
<td>50</td>
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<tr>
<td>Sirolimus (%)</td>
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<td>74</td>
<td>88</td>
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<tr>
<td>Zotarolimus (%)</td>
<td>0</td>
<td>18</td>
<td>12</td>
<td>0.121</td>
</tr>
</tbody>
</table>

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 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 content (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 lead to a marked decrease of
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$(\pm14)$ % of the lumen were covered with endothelial cells 2 weeks after vascular injury. Treatment with rapamycin evoked more consistent re-endothelialization (84 $(\pm7)$%, $P = 0.038$) of the injured arteries. Restoration of EMAP-II, caused a significant decrease in re-endothelialization (42 $(\pm12)$%, $P = 0.005$) 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 CASMC 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 monocytic cells was reduced by rapamycin

Next, we assessed EMAP-II expression in CASMCs and monocytic cells. At baseline, CASMC 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 CASMCs remained higher than in MonoMac6 cells (Figure 6B).
3.7 Rapamycin suppressed increased endothelial-monocyte activating peptide II levels after percutaneous coronary intervention in patients

To further evaluate if the observed regulation of EMAP-II after mechanical injury takes place in patients, EMAP-II protein levels were analysed in blood samples from patients undergoing PCI. In accordance to our in vitro data, we found a significant overall increase in EMAP-II protein (2.21 ± 0.42 to 4.01 ± 0.96 ng/mL) after vessel injury in patients. As PCI in distal coronary artery segments is associated with a higher rate of restenosis and rapamycin-eluting stents display the most beneficial effect in small vessels, we focused on the EMAP-II levels drawn from patients with intervention in distal coronary artery segments. In this patient subgroup, a significant, slightly more pronounced increase was observed whereas intervention of proximal segments did not show an increase (Figure 6C). Multivariate analysis did not reveal an influence of previous myocardial infarction, number of implanted stents or implantation of a sirolimus-eluting stent on EMAP-II expression after PCI in native coronary arteries.

To assess weather oral administration of rapamycin would have an effect on EMAP-II expression in patients, EMAP-II protein was measured in samples from the OSIRIS trial. Starting from comparable baseline values (2.82 ± 0.77 vs. 2.74 ± 1.08 ng/mL), a profound reduction of EMAP-II protein level was observed on the day of PCI (4.21 ± 1.16 vs. 1.57 ± 0.61 ng/mL) after treatment with a loading dose of rapamycin. This suppressive effect was maintained as long as rapamycin was continued (Figure 6D).
4. Discussion

Rapamycin reduces the risk of in-stent restenosis by inhibiting neointimal proliferation in patients. 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 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 CASMCs 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 injury and its inhibition by rapamycin in vivo. The observed expression in subendothelial leukocytes and medial SMCs is in line with its known expression in stimulated monocytes and vascular SMCs. Since the model of injury used in our study leads to induction of medial cell apoptosis, the observed upregulation...
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 cells\(^11\) 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.

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**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 (\(^*P<0.01\), \(^8P<0.05\)). (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 ± 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 ± SEM, \(^*P<0.01\)).
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. Supressing 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, 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.

In human vascular repair, monocytes and neutrophils play a crucial role. EMAP-II is a potent cytokine in this context since it induces migration of monocytes and chemotaxis of neutrophils. 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, 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. 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. However, in this study, extraluminal local drug administration suggests high tissue drug concentrations probably even exceeding previously reported tissue levels. This concurs with results of Farb et al. who reported incomplete neointimal healing of stented areas after high dose oral sirolimus treatment. Furthermore, a high rate of re-endothelialization was observed with low dose everolimus treatment in the same study. 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. 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.

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. 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
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