

# Neointimal Expression of Rapamycin Receptor FK506-Binding Protein FKBP12: Postinjury Animal and Human In-Stent Restenosis Tissue Characteristics

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## Key Words

Dendritic cells · Drug-eluting stents · In-stent restenosis · Rapamycin

## Abstract

Despite excellent clinical results for sirolimus (rapamycin)-eluting stents, the exact mechanisms of antirestenotic activity and affected cellular targets are incompletely understood. Therefore, we determined the presence and temporospatial expression pattern of FKBP12, the primary intracellular receptor of rapamycin, in rat carotid arteries after balloon injury, as well as in human in-stent restenosis and primary stable coronary atheroma. FKBP12 expression was assessed by immunohistochemistry. Rat carotid arteries revealed maximal expression in  $57.7 \pm 4.0\%$  of neointimal cells at day 7. A large proportion of these FKBP12+ cells showed lumenally confined co-expression with dendritic cell markers. Despite a considerably thicker neointima at day 28, presence of FKBP12 decreased ( $8.5 \pm 1.9\%$ ,  $p = 0.02$ ) with a scattered pattern in luminal and deep neointima. Likewise, human in-stent restenosis atherectomy specimens (time after stent implantation 2–12 months) revealed a comparable extent of cellular rapamycin receptor expression ( $9.3 \pm 1.0\%$ ) that significantly differed from that found in primary

stable atheroma ( $1.3 \pm 0.4\%$ ,  $p < 0.001$ ). In conclusion, the rapamycin receptor is predominantly present during early neointima formation, while mature neointimal atheromas show a relatively low expression without confinement to luminal areas. Co-expression of FKBP12 and dendritic cell markers suggests that dendritic cells may be another important target for early and long-term rapamycin effects.

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## Introduction

Sirolimus (rapamycin)-eluting stents show excellent clinical results as to reduction of neointimal hyperplasia and restenosis, also in patients and lesions at high risk of in-stent restenosis (ISR) [1–4]. Long-term observations in larger patient populations demonstrated the persistence of low restenosis rates [5, 6]. Currently, drug-eluting stents are widely used in everyday clinical practice [7].

With the advent of more regulated drug release and next-generation polymer technology [8], a controlled and ‘programmable’ application has become feasible. Although a slightly higher angiographic lumen loss was reported for fast-release rapamycin stents as compared to slow-release coatings [6], little is known about the tempo-

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ral and spatial distribution of potential cellular targets of rapamycin within the stented vessel. Also, despite anti-proliferative and antimigratory effects on vascular smooth muscle (SM) cells [9, 10], additional cellular targets such as immune cells are likely to be present and affected by this immunosuppressive agent. Indeed, inflammatory reactions are apparently related to restenosis development [11–13]. In this context, we could identify dendritic cells (DCs) as a novel cell type in both rat carotid neointima after balloon injury and in human ISR specimens [14, 15].

Rapamycin inhibits several intracellular signaling pathways of cell cycle-regulating TOR (target of rapamycin) proteins by forming a complex with its primary intracellular receptor FK506-binding protein 12 (FKBP12), and targets the cellular protein FKBP12-rapamycin-associated protein/mammalian target of rapamycin/rapamycin and FKBP12 target 1 (FRAP/mTOR/RAFT1), thereby inhibiting or delaying cell cycle progression [16]. Thus, cells containing high levels of FKBP12 should be highly affected by rapamycin.

In the present study, we therefore assessed the presence and temporospatial expression pattern of FKBP12, the primary intracellular receptor of rapamycin, during neointima formation in rat carotid arteries after balloon injury. Also, the cell-bound distribution of FKBP12 in atherectomy samples from human ISR and atherosclerotic primary lesions was investigated. We demonstrate strong expression of FKBP12 especially in early neointimal cells, decreasing expression with ongoing neointima formation, and relatively low expression in ‘mature’ human ISR lesions and primary atherosclerotic plaques.

## Methods

### Rat Carotid Balloon Injury

Male Sprague-Dawley rats (350–400 g) were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (15 mg/kg). Left carotid balloon injury was done as previously described [14]. The right carotid artery was surgically exposed but not injured. After 7 or 28 days, both carotid arteries were harvested (6 rats/time point). Animal investigations conformed with institutional guidelines and were approved by the Committee for Animal Care and Use (Government of Oberbayern, Germany; AZ 211-2531).

### Patients and Human Arterial Specimens

A series of 35 symptomatic patients had been treated by percutaneous directional atherectomy (Danimed, Isernhagen, Germany) [17–19]. A total of 35 coronary atherectomy probes were obtained including 12 ISR lesions (time after stent implantation 2–12 months), and 23 primary target lesions from patients with

**Table 1.** Patients’ characteristics

Variable	Patients with ISR (n = 12)	Patients with chronic stable atheroma (n = 23)	p value
Age, years	58 ± 10	57 ± 10	0.6
Male gender	9 (75)	18 (78)	0.8
Hypertension	6 (50)	12 (52)	0.7
Diabetes	7 (58)	6 (26)	0.1
Smokers	4 (33)	5 (22)	0.5
Hyperlipidemia	6 (50)	6 (26)	0.9
Obesity	4 (33)	8 (35)	0.5
Familial disposition	4 (33)	9 (39)	1.0
Aspirin	12 (100)	23 (100)	–
Statin	11 (92)	20 (87)	0.7
ACE inhibitor	6 (50)	12 (52)	0.9
β-Blocker	9 (75)	17 (74)	0.9

Values in parentheses represent percentage.  
ACE = Angiotensin-converting enzyme.

stable angina (angiographic stenosis degree >75%). Tissue samples were obtained from 22 left anterior descending, 9 right, 2 circumflex coronary arteries, and 2 venous bypass grafts. Age, sex, cardiovascular risk factors, and medication did not significantly differ between both cohorts (table 1). Thirty to 60 min before and during the atherectomy procedure, all patients received heparin intravenously. All patients had given informed consent for subsequent analysis of excised plaque tissue, and tissue collection was carried out under institutional ethics committee-approved protocols.

### Immunohistochemistry

Rat arterial specimens were fixed in buffered 4% formaldehyde without transluminal fixative perfusion in order to preserve the innermost vascular wall layer as well as luminal cell aggregates, as previously described [14]. Immunohistochemistry was done in 4-μm paraffin sections after proteolysis with Target Unmasking Fluid (PanPath). Slides were blocked by fetal calf serum at a dilution of 1:25 for FKBP12, S100 and OX-62 in rats and 1:5 for FKBP12 in human samples [15, 19]. A polyclonal rabbit anti-FKBP12 antibody (1:400; Biomol; catalog No. SA218) was used for FKBP12 detection. Other antibodies used in the present study were polyclonal rabbit anti-S100 (1:100; Sigma, catalog No. S2644) as well as monoclonal mouse anti-rat DC (1:20; Pharmingen; clone OX-62) and mouse anti-α-SM actin (1:75; Boehringer; clone asm-1). For polyclonal antibodies, AffiniPure mouse anti-rabbit IgG (1:75; Dianova; code No. 211-005-109) was applied following primary antibody incubation. Primary antibodies were visualized with the APAAP technique (Boehringer) and Fast Red (Sigma); nuclei were counterstained with hematoxylin. Double staining was done for FKBP12/OX-62 in rats as previously described [14]. Briefly, after OX-62 immunostaining with the APAAP method and Fast Blue (Sigma) as chromogen, tissue sections were washed in phosphate-buffered saline, and consecutively incubat-

ed with FKBP12 antibodies. Thereafter, AffiniPure mouse anti-rabbit IgG was applied. FKBP12 antibodies were visualized by APAAP and Fast Red. To improve the detection of Fast Blue immunostaining, hematoxylin nuclear staining was omitted. Rat carotid sections without primary antibody incubation, as well as rat myocardial sections served as negative controls in each staining procedure, as previously described [14]. In addition, negative controls for human specimens included omission of the antibodies and staining with unspecific antibody (mouse monoclonal IgG1, clone NCG01, Dianova and ChromPure rabbit IgG, code No. 011-000-003, Dianova, respectively) in the same concentrations as the primary antibodies, as reported previously [15, 19].

#### *Morphometric Analysis*

A computer-assisted morphometric system (VFG-1-grafic card/VIBAM 0.0 Software) was used to automatically count labeled nuclei per defined area [14, 15, 20, 21]. Area, cellularity and the percentage of immunohistochemically labeled cells were evaluated from six randomly selected fields per tissue sample cross section. The percentage of labeled cells was expressed as the mean number of positive cells per mean total number of cells for each layer [14, 15, 19].

#### *Statistical Analysis*

Data are reported as expression [percentage (mean  $\pm$  SEM) of stained cells per total number of cells] as well as median and range. We assessed group differences by use of  $\chi^2$  test for categorical variables. Probability was calculated with the Mann-Whitney U test for differences in cell density and expression of intimal determinants. p values of  $<0.05$  were considered to denote statistical significance.

## **Results**

### *Neointima Formation in Injured Rat Carotid Arteries*

Rat carotid arteries were analyzed 7 and 28 days after balloon injury. At day 7, one to two layers of neointimal cells were observed (fig. 1a, b). FKBP12, the intracellular receptor of rapamycin, was strongly expressed in neointimal cells at this time point, as shown in figure 1a ( $57.7 \pm 4.0\%$  of all neointimal cells, median 60.0%, range 28.2%). A large proportion of neointimal cells were identified as DCs due to intense immunolabeling with S100 and OX-62 ( $63.2 \pm 5.0\%$ , median 62.0%, range 36.8%, and  $65.0 \pm 5.7\%$ , median 62.2%, range 31.7%, respectively). Co-expression of these DC markers and FKBP12 was detected, as shown in figure 1b. Pronounced FKBP12 expression and DC immunostaining were confined to the neointima, while  $\alpha$ -SM-actin+ SM cells were present in the neointima, media and adventitia (not shown). At day 28, following arterial injury, FKBP12 revealed a sparse, scattered label both at the lumen and throughout the neointima, beyond an overall decreased expression as compared to day 7 ( $8.5 \pm 1.9\%$ , median 8.5%, range 9.6%,

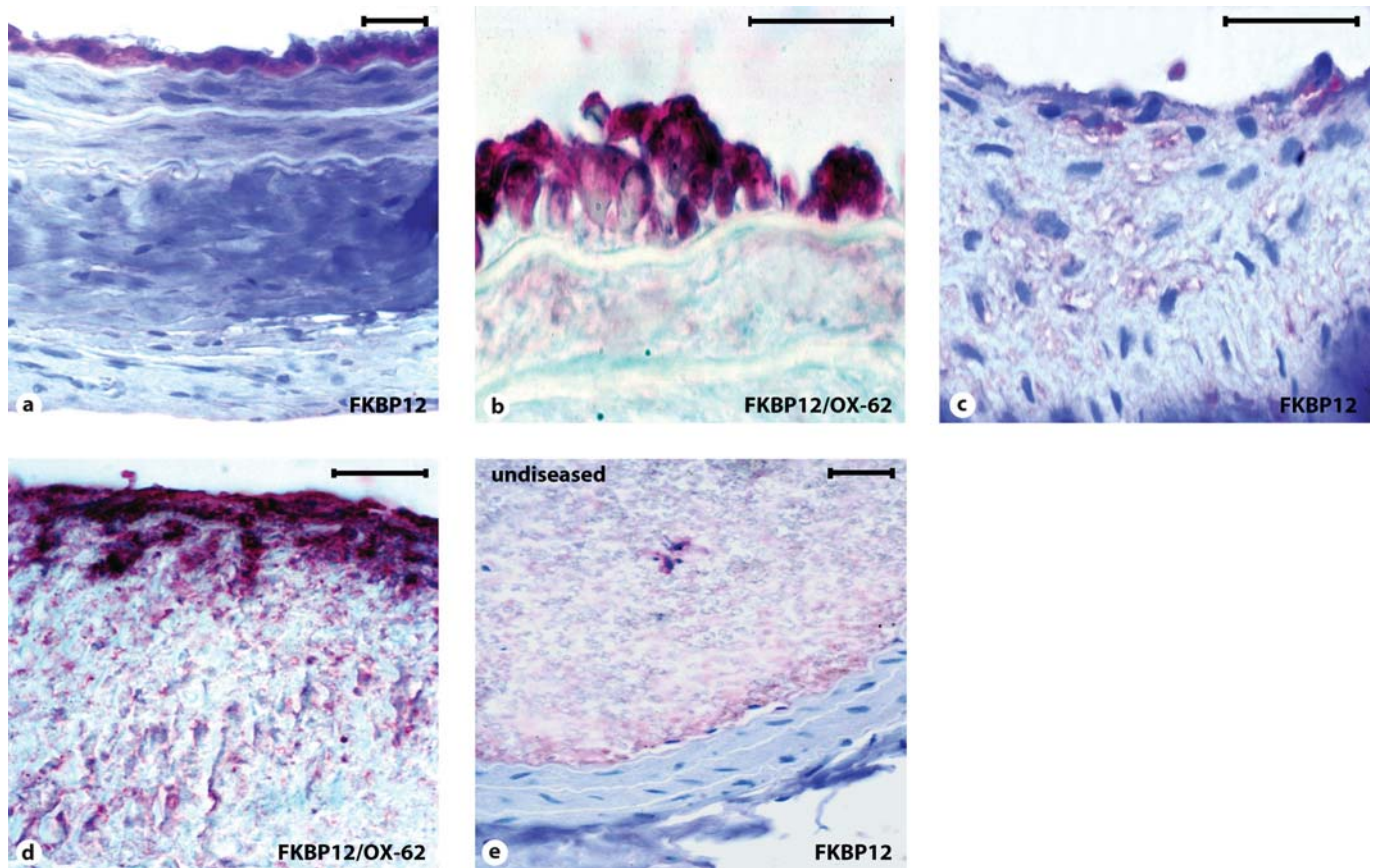
$p = 0.02$ ; fig. 1c). At this time point, the neointimal compartment had increased  $>10$ -fold exhibiting a distinct luminal layer of cells labeled by DC markers (S100:  $1.9 \pm 0.6\%$ , median 2.4%, range 4.1%; OX-62:  $1.5 \pm 0.6\%$ , median 1.1%, range 3.9%; each  $p < 0.01$  vs. day 7). Again, distinct co-expression of DC markers and FKBP12 was seen (fig. 1d).  $\alpha$ -SM actin immunoreactivity was found in all vascular wall layers (not shown). Nontraumatized contralateral carotid artery segments did not show any immunostaining for FKBP12 (fig. 1e).

### *Human ISR and Primary Atherosclerotic Plaques*

Consistently, ISR tissue from all 12 patients showed markedly higher cellularity in ISR compared to the 23 primary lesions ( $763 \pm 13$  vs.  $309 \pm 36$  cells/mm<sup>2</sup>,  $p = 0.004$ ). FKBP12 signaling was found in all ISR lesions and in the majority of primary lesions. The percentage of neointimal cells stained by FKBP12 was  $9.3 \pm 1.0\%$  in ISR lesions (median 8.6, range 11.1). Positive cells were found within all tissue areas, thereby revealing a scattered pattern without persistent luminal confinement (fig. 2a). In contrast, FKBP12 immunolabeling was low with  $1.3 \pm 0.4\%$  in primary atheroma (median 0.4%, range 5.0%,  $p < 0.001$ ) (fig. 2b). Expression of intimal rapamycin receptor did not show a specific predilection site, as found for ISR tissue. Notably, some atherectomy samples demonstrated luminal presence of distinctive FKBP12-positive cells, irrespective of ISR or primary lesion type. Interestingly, there was no significant difference between FKBP12 expression in ISR tissue from coronary arteries and from diseased vein grafts. In addition, there was no relationship between expression and the time course after stent implantation.

## **Discussion**

This study addressed the expression of FKBP12, the primary intracellular receptor of rapamycin, during neointima formation in rat carotid arteries as well as in human ISR and primary target lesions. We herein demonstrate (1) that the rapamycin receptor is strongly prominent in neointimal cells with a maximum at day 7, (2) pronounced FKBP12 expression by neointimal DCs, and (3) only a sparse, scattered label both at the lumen and throughout the neointima at day 28 in the rat model. Furthermore, we found (4) a comparable extent of these cells within human ISR and (5) a significantly lower expression level in chronic stable atheroma.

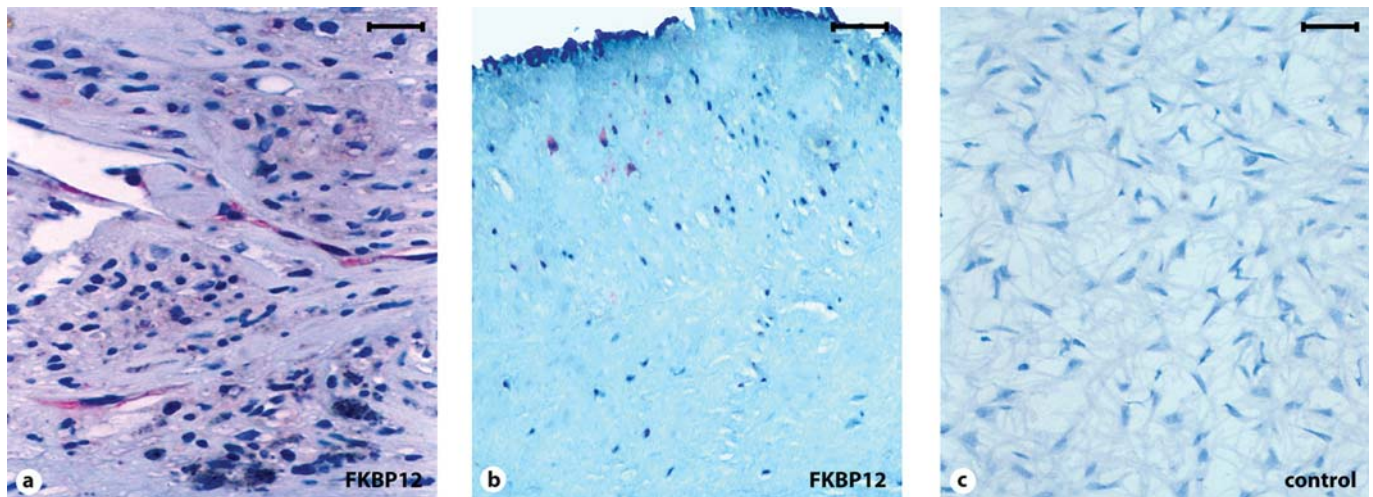


**Fig. 1.** The presence and distribution of FKBP12, the primary intracellular receptor of rapamycin, are shown. **a** Rat carotid artery at day 7 after balloon injury. FKBP12 is strongly expressed in early neointimal cells adhering to the lamina elastica interna. Note absence of signals in media and adventitia. **b** Double staining for FKBP12 (red) and OX-62 as DC marker, illustrating the colocalization of both determinants in adherent neointimal cells. **c** Hyperplastic rat carotid neointima at day 28. Detection of

FKBP12 labeling in the luminal layer as well as in sparsely scattered cells throughout the neointima. **d** Double staining for FKBP12 (red) and OX-62 as DC marker, illustrating strong colocalization of both determinants in luminal neointima. **e** Luminal, nontraumatized control segment without signals of FKBP12 in the vessel wall and with single luminal signals without intimal adherence. Bar = 25  $\mu$ m.

Our present findings on FKBP12 immunolabeling extend previous work on the detection of FKBP12 in human atheroma [20]. Herein, Zohlh ofer et al. [20] demonstrated signals of FKBP12 in (late) carotid restenotic neointima. Expression was seen in the cytoplasm of occasional SM cells, while none was observed in the undiseased media. Given the early maximum of neointimal and transmural FKBP12 expression in the rat model, and decreasing expression at later time points (fig. 1), our current observations point to an early time frame for rapamycin-mediated effects during postangioplasty remodeling processes. In consequence, and in view of only a residual, scattered presence of FKBP12+ cells in 28-day rat lesions (fig. 1c, d) and in 'late' human neointima

(fig. 2), this body of data favors the early therapeutic application of rapamycin from the endoluminal site where the maximal receptor density can be anticipated (fig. 1a, b). Importantly, our present observations highlight neointimal DCs to represent an important cell type exhibiting FKBP12 binding sites, and show only a scattered expression by some non-DCs, putatively luminal endothelial cells and SM cells scattered within the deep neointima (fig. 1b, d). Therefore, we would like to propose that the marked prevention of restenosis by use of rapamycin-eluting stents, as widely shown, is in large part mediated by effects on early neointimal DCs. Accordingly, rapamycin specifically induced apoptosis in monocyte- and CD34-derived DCs, but not in monocytes and macro-



**Fig. 2.** Human coronary atherectomy samples. **a** ISR tissue obtained from the left anterior descending coronary artery of a 62-year-old man 6 months after stent implantation. The ISR lesion demonstrates a percentage of FKBP12-positive cells scattered throughout the neointima. **b** Primary coronary plaque from a 58-

year-old patient with exercise-induced stable angina. Note that only a few signals of FKBP 12 were commonly observed in primary atherosclerosis. **c** ISR tissue stained with unspecific mouse IgG1 as negative control to exclude nonspecific immunostaining. Bar = 50  $\mu$ m.

phages [21]. Taken together, these data suggest that clinical restenosis prevention by use of rapamycin-eluting stents may be attributed, among other mechanisms such as antiproliferative effects, to the induction of DC apoptosis at early postinterventional time points [9].

Our data on 'late' postinjury stenosis, ISR and chronic lesions consistently demonstrate a scattered pattern of FKBP12+ cells throughout the neointima, having lost their initial luminal predilection site (fig. 1c, d, 2). As a consequence, any growth-inhibitory effects by rapamycin on these 'late' lesions is unlikely due to the embedment of target cells in solid neointima. However, we also observed some luminal cells that exhibit distinctive signaling for the rapamycin receptor in these 'late' lesions, putatively of dendritic and/or endothelial cell origin [15]. Therefore, we would like to suggest that these luminal

cells may respond to rapamycin, when drug-eluting stents are implanted in coronary segments. Given the long-term endothelial dysfunction – shown only for sirolimus-eluting stents [22, 23] – and the late attenuation of collateral function following drug-eluting stent placement as previously reported for both sirolimus and paclitaxel [24], it is conceivable that the lumenally located receptor-positive cells may be targeted by rapamycin that could induce endothelial dysfunction adjacent to the stent or distant angiogenesis.

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