Selective Clearance of Macrophages in Atherosclerotic Plaques by Autophagy

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Objectives
The purpose of this study was to investigate whether stent-based delivery of an inhibitor of mammalian target of rapamycin (mTOR) can selectively clear macrophages in rabbit atherosclerotic plaques.

Background
Current pharmacologic approaches to stabilize atherosclerotic plaques have only partially reduced the incidence of acute coronary syndromes and sudden death. Macrophages play a pivotal role in plaque destabilization, whereas smooth muscle cells (SMC) promote plaque stability.

Methods
Stents eluting the mTOR inhibitor everolimus were implanted in atherosclerotic arteries of cholesterol-fed rabbits. In addition, in vitro experiments using explanted atherosclerotic segments and cultured macrophages as well as SMC were performed.

Results
Stents eluting everolimus led to a marked reduction in macrophage content without altering the amount of SMC compared with polymer control stents. In vitro studies showed that everolimus treatment induced inhibition of translation in both cultured macrophages and SMC. However, cell death occurred only in macrophages and was characterized by bulk degradation of long-lived proteins, processing of microtubule-associated protein light chain 3, and cytoplasmic vacuolization, which are all markers of autophagy. Everolimus-induced autophagy was mediated by mTOR inhibition, because cell viability was not affected using tacrolimus, an mTOR-independent everolimus analog. Moreover, mTOR gene silencing was associated with selective induction of macrophage cell death. Autophagic macrophage cell death was confirmed by transmission electron microscopy both in cultured cells and in atherosclerotic explants.

Conclusions
Stent-based delivery of everolimus selectively cleared macrophages in rabbit atherosclerotic plaques by autophagy, an mTOR inhibition-dependent and novel mechanism to induce cell death in mammalian cells. (J Am Coll Cardiol 2007;49:706–15) © 2007 by the American College of Cardiology Foundation

Atherosclerotic plaque destabilization and rupture are one of the main causal events of acute coronary syndromes, including myocardial infarctions (1,2). On angiography, “vulnerable” rupture-prone plaques are often not considered to be at risk, because they seem not to be flow limiting before destabilization occurs (3). Macrophages, which are an essential component of such plaques, play a pivotal role in the destabilization process, whereas smooth muscle cells (SMC) contribute to plaque stability (4). As a consequence, it is generally assumed that macrophage removal stabilizes the plaque (5). Yet, mechanisms whereby macrophages can be eliminated from plaques without influencing other cell types, including SMC, are unknown. Systemic therapy with statins has been shown to reduce but not eliminate macrophages from atherosclerotic plaques (6). Suzuki et al. (7) reported that stent-based delivery of rapamycin (sirolimus), a potent mammalian target of rapamycin (mTOR) inhibitor, had a profound effect on inflammatory cell activity and cytokine release in nonatherosclerotic porcine arteries. In the present study, we investigated whether implantation of stents eluting everolimus [40-O-(2-hydroxyethyl)-rapamycin], a rapamycin derivative, could selectively clear macrophages from atherosclerotic plaques.
Methods

In vivo experiments. Twelve New Zealand White rabbits (Merelbeke, Belgium) were fed a 0.3% cholesterol-supplemented diet for 40 weeks to induce atherosclerotic plaques in the aorta (8). Experiments were approved by the local ethical committee. Procedures were performed on anesthetized rabbits using sterile techniques. At the index procedure, the marginal ear vein was cannulated, and the rabbit was anesthetized with sodium pentobarbital (30 mg/kg intravenously). The right carotid artery was dissected under sterile conditions and a 6F sheath was introduced. Under fluoroscopy, a 0.014-inch guide wire (Guidant Whisper, Advanced Cardiovascular Systems, Santa Clara, California) was positioned in the right or left iliac artery, and a single dose of aspirin (60 mg/kg) as well as heparin (100 IU/kg) was administered. Two stainless steel stents (Guidant Multi-Link Penta, 3.5/13 mm) coated with either everolimus or durable polymer only were deployed (1 inflation at 9 atm during 15 to 20 s) in the infrarenal aorta of each animal after randomization. Stents were deployed at a minimum distance of 1 cm from each other. All rabbits were continued on 0.3% cholesterol-supplemented diet for 4 weeks to evaluate the response to drug-eluting stents.

The rabbits were then killed, the aortas were flushed with saline, and all sections were harvested and placed in 4% formalin. The specimens were immersion-fixed for 24 h before processing. Stented vessels underwent plastic embedding (1:1 butyl methacrylate:methyl methacrylate; Technovit), and sections were obtained by saw-and-grinding using standard techniques. Stented segments were cut into one midsection. Nonstented segments were embedded in paraffin blocks for processing. All specimens were stained with hematoxylin-eosin for histopathologic analysis. Immunohistochemical detection of macrophages (RAM-11, dilution 1:1000; Dako, Glostrup, Denmark) and SMC (monoclonal anti-α-smooth muscle actin, dilution 1:2000; Sigma, St. Louis, Missouri) were carried out by the indirect peroxidase antibody conjugate technique (9–11). The sections were counterstained with 0.2% Triton X-100, 10% trichloroacetic acid, resuspended in 0.2 N NaOH, and measured by liquid scintillation counting.

For mTOR gene silencing experiments, cells were transfected with siRNA (Dharmacon, Lafayette, Colorado) according to the instructions of the manufacturer. To examine de novo protein synthesis, cells were treated with everolimus (10 μmol/l) or cycloheximide (10 μg/ml) for 4 hours, and pulse-labeled for 1 h at 37°C with 5 μCi Pro-mix L-[^35S]甲基) in vitro cell labeling mix (Amersham Biosciences, United Kingdom) in cysteine/methionine-free DMEM. After homogenization of cells in hypotonic lysis buffer (10 mmol/l Tris, 1 mmol/l EDTA, 0.2% Triton X-100), labeled proteins were precipitated with 10% trichloroacetic acid, resuspended in 0.2 N NaOH, and measured by liquid scintillation counting.

Analysis was performed by two experienced and blinded operators. The percentage of the stent strut circumference surrounded by macrophages or SMC was assessed for each single strut, using RAM-11 or α-SMC actin staining respectively. The RAM-11 positive area in the plaque was measured for all stented and nonstented segments.

In vitro experiments, cell culture. The murine macrophage cell line J774A.1 was grown in RPMI 1640 medium (Invitrogen, San Diego, California) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 20 U/ml polymyxin B, and 10% fetal bovine serum. Alternatively, peritoneal macrophages were isolated 4 days after injection of Brewer’s thioglycolate medium into the peritoneal cavity of C56BL/6 mice as reported previously (12). The SMC were isolated from mouse or rabbit aorta by collagenase type 2 (Worthington, Lakewood, New Jersey) and elastase (Sigma) digestion (60 to 90 min at 37°C) at 300 U/ml and 5 U/ml final concentration, respectively, and cultured in F10 Ham medium supplemented with 10% fetal bovine serum and antibiotics. Evaluation of cell viability before and after everolimus (10 μmol/l), tacrolimus (10 μmol/l; Alexis, Lausen, Switzerland), or cycloheximide (10 μg/ml; Sigma) was based on the incorporation of the supravital dye neutral red by viable cells (13). The activity of tacrolimus was evaluated by its ability to decrease nitrite production by LPS-stimulated J774 macrophages (14). To examine de novo protein synthesis, cells were treated with everolimus (10 μmol/l) or cycloheximide (10 μg/ml) for 4 hours, and pulse-labeled for 1 h at 37°C with 5 μCi Pro-mix L-[^35S]甲基) in vitro cell labeling mix (Amersham Biosciences, United Kingdom) in cysteine/methionine-free DMEM. After homogenization of cells in hypotonic lysis buffer (10 mmol/l Tris, 1 mmol/l EDTA, 0.2% Triton X-100), labeled proteins were precipitated with 10% trichloroacetic acid, resuspended in 0.2 N NaOH, and measured by liquid scintillation counting.

Degradation of long-lived proteins was determined in serum-containing medium according to a method previously reported (15). For DNA fragmentation assays, cul-
cultured cells (10⁶) were lysed in 0.5 ml hypotonic lysis buffer supplemented with 250 μg proteinase K. Lysates were incubated for 1 h at 50°C, then supplemented with 5-μl volumes of DNase-free RNase A (2 mg/ml) and incubated for an additional hour at 37°C. The samples were precipitated overnight with 1/10 volume of 3 mol/l sodium acetate and 1 volume of isopropanol. The DNA pellets were air dried and dissolved in TE buffer (10 mmol/l Tris, 1 mmol/l EDTA, pH 7.4). After electrophoresis in 2% agarose, DNA laddering was visualized under UV light by staining the agarose gel with ethidium bromide.

For Western blot analyses, cultured cells were lysed in an appropriate volume of Læmmli sample buffer (Bio-Rad; Hercules, California). Cell lysates were then heat-denatured for 4 minutes in boiling water and loaded on a SDS polyacrylamide gel. After electrophoresis, proteins were transferred to an Immobilon-P Transfer Membrane (Millipore; Bedford, United Kingdom) according to standard procedures. Membranes were blocked in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and 5% nonfat dry milk (Bio-Rad) for 1 h. After blocking, membranes were probed overnight at 4°C with primary antibodies in antibody dilution buffer (TBS-T containing 1% nonfat dry milk), followed by 1 h of incubation with secondary antibody at room temperature. Antibody detection was accomplished with SuperSignal West Pico or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, Illinois) using a Lumi-Imager (Roche, Mannheim, Germany).

The following mouse monoclonal primary antibodies were used: anti–caspase-3 (clone 19) from BD Transduction Laboratories (Lexington, Kentucky) and anti–β-actin (clone AC-15) from Sigma. Rabbit polyclonal antibodies included anti–FK506-binding protein 12 (FKBP12) (Abcam, Cambridge, United Kingdom), anti–mTOR, anti–phospho-mTOR (Ser2448), anti–p70 S6 kinase, anti–phospho-p70 S6 kinase (Thr389), anti–phospho-p70 S6 kinase (Thr421/Ser424), anti–4E-binding protein 1 (4EBP1), anti–phospho–4E-BP1 (Thr37/46), anti–eukaryotic initiation factor 2α (eIF2α), anti–phospho-eIF2α (Ser51), anti–eukaryotic elongation factor 2 (eEF2), anti–phospho–eEF2 (Thr56), and anti–cleaved caspase-3 from Cell Signaling Technology (Beverly, Massachusetts). Rat anti–microtubule-associated protein light chain 3 (LC3) polyclonal antibody raised against the synthetic peptide H2N-PSDRPFKQRRSFADC-CONH2 was prepared by Eurogentec (Seraing, Belgium) and affinity purified on an immobilized peptide-Sepharose column. Peroxidase-conjugated secondary antibodies were purchased from Dako.

EXPLANTS. Four New Zealand white rabbits were fed a cholesterol-rich (1.5%) diet for 14 days. To induce atherosclerotic lesions, a silicone collar (16) was positioned for 14 days around the carotid arteries while continuing the cholesterol diet (17). Subsequently, ring segments of these cuffed arteries were incubated in serum-free F10 Ham medium at 37°C with or without everolimus (10 μmol/l; Novartis, Basel, Switzerland). Everolimus was stored light protected at −20°C as a 10 mmol/l stock solution in ethanol. We used only fresh dilutions of the everolimus stock in serum-free culture medium. Because everolimus is unstable in aqueous solution, culture medium containing everolimus was refreshed every 12 h. After 3 days, the ring segments were prepared for transmission electron microscopy.

Transmission electron microscopy. Cultured macrophages, SMC, or explanted atherosclerotic segments were fixed in 0.1 mol/l sodium cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde solution for 2 h, then rinsed (3 × 10 minutes) in 0.1 mol/l sodium cacodylate-buffered (pH 7.4) 7.5% saccharose and postfixed in 1% OsO4 solution for 1 hour. After dehydration in an ethanol gradient (70% ethanol for 20 min, 96% ethanol for 20 min, 100% ethanol for 2 × 20 min), samples were embedded in Durcupan ACM. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined in a Philips CM 10 microscope at 80 kV.

Statistical analysis. Data are expressed as mean ± SEM. The percentage strut circumference surrounded by macrophages or SMC was compared between polymer control- and everolimus-stented arteries by using the two-tailed unpaired Student t test. The RAM-11–positive area in the plaque, de novo protein synthesis, cell viability, and degradation of long-lived proteins, and the data of the in vitro effect of everolimus on the mTOR pathway and protein synthesis in cultured macrophages and SMC were compared among groups by using one-way analysis of variance (ANOVA) followed by the Dunnett test. If the variances were unequal (Levene test for homogeneity of variances; p < 0.05) then logarithmically transformed values (with homogenous variances) were analyzed. The relative expression of mTOR mRNA and the viability of macrophages and SMC were compared between mTOR siRNA- and siControl-treated cells by using unpaired Student t test. The SPSS software package 12.0 (SPSS Inc., Chicago, Illinois) was used for these purposes.

Results
In vivo experiments. Polymer control and everolimus-eluting stents were implanted in atherosclerotic arteries of cholesterol-fed rabbits. After 1 month, plaques treated with polymer control stents were characterized by the presence of macrophages and SMC (Figs. 1A, 1C, and 1E). In contrast, in plaques treated with everolimus-eluting stents the macropage content around the stent struts was significantly (>90%) reduced without changing the smooth muscle cell content (Figs. 1B, 1D, and 1E). Moreover, macrophages were also effectively cleared throughout the atherosclerotic plaque (Fig. 1F).
In vitro experiments. Western blot experiments showed that macrophages and SMC contained similar amounts of the everolimus receptor protein FKBP12 as well as phosphorylated (Ser2448) and total mTOR, which binds the everolimus-FKBP12 complex (Fig. 2A). Everolimus treatment resulted in dephosphorylation of the downstream mTOR targets p70 S6 kinase and 4E-BP1, whereas eIF2/β and eEF2 were hyperphosphorylated (Fig. 2B). Quantitative data are presented in Table 1. Everolimus also inhibited de novo protein synthesis (Fig. 2C). Viability of macrophages decreased rapidly by everolimus treatment in contrast to SMC (Figs. 3A and 3B). Administration of tacrolimus did not result in macrophage cell death in culture (Figs. 3C and 3D). Massive cell type-specific initiation of cell

![Image](https://via.placeholder.com/150)
death was obtained not only with everolimus but also with the translation inhibitor cycloheximide (Figs. 3E and 3F). To ensure an involvement of mTOR in everolimus-induced cell death, gene-silencing experiments with mTOR-specific siRNA were performed. Down-regulation of mTOR gene expression was demonstrated at the mRNA (Fig. 4A) and protein (Fig. 4B) levels in both cell types after transfection with mTOR-specific siRNA but not with siControl nontargeting siRNA. The mTOR gene silencing was associated with selective induction of macrophage cell death (Fig. 4C), indicating that mTOR is the primary target of everolimus, leading to macrophage-specific cell death.

Cycloheximide-induced macrophage cell death was associated with cleavage of caspase-3 and internucleosomal DNA fragmentation (Fig. 5A). However, the type of cell death induced by everolimus was not characterized by these features, notwithstanding that levels of procaspase-3 in macrophages decreased during treatment (Fig. 5A). Bulk degradation of long-lived proteins occurred in macrophages after treatment with everolimus but not in SMC (Fig. 5B). Moreover, everolimus-treated macrophages showed conversion of the 18-kDa protein LC3-I into the 16-kDa protein LC3-II (Fig. 5C), resulting in an increased LC3-II/LC3-I ratio. The ratio of LC3-II to LC3-I in SMC remained unaffected during everolimus administration. Nonetheless, SMC underwent bulk degradation of long-lived proteins, as well as processing of LC3 in response to amino acid deprivation by using Earle's Balanced Salt Solution (EBSS) (Figs. 5B and 5C).

Transmission electron microscopy. Everolimus-treated macrophages in culture showed an intact nonpyknotic nucleus and numerous vacuoles in the cytoplasm (Figs. 6B to 6D), whereas control macrophages did not display vacuolization (Fig. 6A). Transmission electron microscopy of everolimus-treated macrophages in explanted atherosclerotic segments derived from collar-treated rabbit carotid arteries showed cell shrinkage, depletion of organelles, and presence of large autophagosomes containing membranous whorls and remnants of cytoplasmic material (Figs. 7A and 7B). These findings were not observed in everolimus-treated SMC, either in culture (Figs. 6E and 6F) or in explanted atherosclerotic segments (Figs. 7C and 7D).

Discussion

The major finding of this study is that mTOR inhibition by everolimus led to a selective removal of macrophages within rabbit atherosclerotic plaques without influencing the viability of SMC. This observation is important in the context of plaque stabilization, because it is generally assumed that presence of macrophages triggers plaque destabilization (5). Although it has been reported that statins can reduce macrophages in atherosclerotic plaques (6), they do not reduce mortality for several months (18). Therefore, there is...
still a need for adequate local interventional therapies, which make it possible to “buy time” until systemic therapies confer significant protection (18).

Our in vivo results showed that everolimus-eluting stents selectively cleared macrophages both around the stent struts and throughout the atherosclerotic plaque, the latter being...
likely attributed to the lipophilic properties of everolimus, because the drug may pass easily through cell membranes, enabling mural distribution.

The mechanism of selective macrophage clearance by everolimus-eluting stents was subsequently unraveled in in vitro experiments. Because mTOR controls translation (19), the effect of everolimus on the protein synthesis effectors p70 S6 kinase, eIF2α, and eEF2 was examined in both macrophages and SMC. These experiments showed a dephosphorylation of p70 S6 kinase and 4E-BP1 as well as a hyperphosphorylation of eIF2α and eEF2, all strongly indicating inhibition of protein translation via mTOR inhibition. Moreover, de novo protein synthesis was significantly inhibited by everolimus. These effects occurred in both macrophages and SMC. However, in contrast to SMC, the viability of macrophages rapidly decreased, indicating a cell type-specific initiation of cell death.

Everolimus binds with high affinity to FKBP12; this complex subsequently binds to mTOR, inhibiting its function. To examine whether the observed induction of cell death in macrophages by everolimus is related to the inhibition of mTOR and is not merely a consequence of binding to FKBP12, additional experiments with tacrolimus (FK506; a rapamycin analog) were performed. Although tacrolimus also binds to FKBP12, only the everolimus-FKBP12 complex but not the tacrolimus-FKBP12 complex...
is a highly specific inhibitor of mTOR (20). Tacrolimus, at the same concentration as everolimus, did not result in macrophage cell death in culture, confirming that the induction of cell death in macrophages by everolimus is a consequence of mTOR inhibition. This is in accordance with a recent report showing that in vivo treatment with tacrolimus did not affect the amount of macrophages in atherosclerotic plaques of collared apoE<sup>−/−</sup> mice, either in de novo (developing) atherosclerosis or in pre-existing lesions (21). Additional evidence for an involvement of mTOR in everolimus-induced cell death was provided by gene-silencing experiments with mTOR-specific siRNA. The mTOR gene silencing was associated with selective induction of macrophage cell death, further indicating that mTOR is the primary target of everolimus leading to macrophage-specific cell death.

Massive cell type-specific initiation of cell death was obtained not only with everolimus but also with the translation inhibitor cycloheximide, suggesting that inhibition of protein translation suppresses normal macrophage function, which in turn leads to macrophage cell death. In contrast, it has been reported that inhibition of translation in SMC induces a modulation towards a differentiated, quiescent, contractile phenotype (22) which renders the cells less sensitive to cell death mediated by inhibition of protein translation. In this context, it is important to note that macrophages are metabolically highly active whereas SMC are not. Cycloheximide-induced macrophage cell death corresponded with apoptosis, as shown by cleavage of caspase-3 and internucleosomal DNA fragmentation. However, the type of cell death induced by everolimus was not characterized by these apoptotic features, notwithstanding that levels of procaspase-3 in macrophages decreased during treatment as a result of nonspecific protein degradation. Therefore, induction of nonapoptotic cell death such as autophagy was considered.
Autophagy is a major intracellular degradation process ubiquitous in eukaryotic cells. In normal conditions, autophagy contributes to the turnover of cellular components by delivering portions of the cytoplasm and organelles to lysosomes (23). Continuous stimulation of this cellular degradation process leads to autophagic cell destruction, also known as type II programmed cell death (23,24). Induction of autophagic cell death by inhibition of mTOR is tightly controlled in eukaryotic cells. Experiments in yeast showed that the TOR pathway regulates phosphorylation of Atg13, a protein important for autophagy (25). The Atg13 protein is phosphorylated in healthy cells but rapidly dephosphorylated upon inhibition of TOR, stimulating its affinity to Atg1.

Autophagy was detected by bulk degradation of long-lived proteins in everolimus-treated macrophages but not in SMC and by an increased LC3-II/LC3-I ratio, a reliable marker of autophagosome formation (26). The ratio of LC3-II to LC3-I in SMC remained unaffected during everolimus administration, suggesting absence of autophagy. These biochemical hallmarks of autophagy were confirmed by transmission electron microscopy, which is considered the gold standard for morphologic assessment of autophagy (26). Autophagic features include an intact non-pyknotic nucleus and numerous vacuoles in the cytoplasm. Similar findings were observed in explanted atherosclerotic segments derived from collar-treated rabbit carotid arteries incubated with everolimus.

The consequences of autophagy in macrophages of human atherosclerotic plaques are currently unknown. How-
ever, autophagy rather than apoptosis is likely to be the preferred type of cell death to clear macrophages from atherosclerotic plaques. Massive apoptotic cell death of macrophages is associated with depletion of phagocytes and with the production of a large number of apoptotic bodies in the atherosclerotic plaque. The accumulation of apoptotic bodies in a condition of defective phagocytic clearance eventually results in secondary necrosis of the apoptotic bodies, which contributes to necrotic core formation, inflammation, and thrombosis. These phenomena may further promote plaque instability and increase the risk of acute atherothrombotic clinical events (27).

Although experimental plaques do not represent a human “vulnerable” situation, the present results are novel in that the drug-induced autophagy in macrophages is an additional strength of stents eluting an mTOR inhibitor, on top of their well-described reduced rate of in-stent restenosis (28), the latter being beyond the scope of the present study.

In conclusion, mTOR inhibition by everolimus leads to a selective, clean, and safe removal of macrophages within the atherosclerotic plaque without influencing the viability of SMC. This selective clearance of macrophages occurred by autophagy due to everolimus-induced inhibition of mTOR, which is a novel mechanism to induce cell death in mammalian cells. Therefore, stent-based delivery of an mTOR inhibitor is a promising method to selectively remove macrophages from the atherosclerotic plaque without affecting viability of SMC, favoring plaque stability.

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