Transcriptional Effects of mTOR Inhibitors on Vascular Smooth Muscle Cells

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Abstract

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Introduction: Cardiac allograft vasculopathy (CAV) is a rapidly progressing arteriosclerotic disease in heart transplant recipients and the major cause of late graft failure. A characteristic feature of CAV is intimal hyperplasia of coronary arteries due to vascular smooth muscle cell (VSMC) proliferation. Currently, the only treatment available to prevent CAV is mechanistic target of rapamycin (mTOR) inhibitors. However, little is known about their mechanism of action in VSMCs beyond signaling alterations. The aim of this study was to determine the transcriptional effects of mTOR inhibitors in VSMCs. Candidate transcripts were identified by a microarray analysis of human coronary arteries transplanted to immunodeficient mice and treated with mTOR inhibitors in vivo. This genome-wide approach identified two major groups of proliferation- and inflammation-related genes that were downregulated by mTOR blockade. We investigated if selected transcripts were mTOR-dependent in model experimental systems of cultured coronary arteries and VSMCs.

Materials and methods: Human coronary arteries and VSMCs were cultured in vitro and treated with the mTOR inhibitor rapamycin at various concentrations and durations. Cultured cells were also treated with tumor necrosis factor (TNF) to induce pro-inflammatory responses similar to known NFκB activation in arteries immediately after transplantation and during organ culture. Real-time quantitative PCR was used to evaluate the effects on gene expression.

Results: In cultured VSMCs, the cell proliferation genes *CCNA2*, *HIST1H3G*, *MKI67*, and *TOP2A* and the inflammatory gene *TLR3* were downregulated by rapamycin, with a peak effect after 24 hours of treatment. In contrast, the inflammatory chemokines *CXCL9* and *CXCL10* were upregulated by rapamycin in TNF-treated VSMCs with a peak effect after 72 hours of treatment, contradictory to the microarray results. In cultured coronary arteries, downregulation was observed for *MKI67* and *TOP2A* and the inflammatory genes *IFI30* and *SPP1* after 72 hours of rapamycin treatment.

Conclusions: The regulation of several cell proliferation genes by mTOR in cultured arteries and cells is consistent with the microarray analysis of coronary artery grafts in humanized mice, and suggests that the in vitro models can be used to further study mechanisms of transcriptional control by mTOR inhibitors in VSMCs. The weak and inconsistent effects observed on inflammatory gene expression in vitro may indicate false positive results of the microarray analysis, although an alternative explanation is that de-differentiation of cultured arteries and VSMCs is not suitable for this aspect of mTOR biology and that further in vivo studies are required.

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Abbreviations

ACTB	Actin, beta
ANOVA	Analysis of variance
BSA	Bovine serum albumin
EC	Endothelial cell
ECM	Extracellular matrix
FBS	Fetal bovine serum
FKBP12	FK506-binding protein 12
CAV	Cardiac allograft vasculopathy
CCNA2	Cyclin A2
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CD40	Cluster of differentiation 40
CDC2	Cell division cycle 2
CDK2	Cyclin-dependent kinase 2
cDNA	Complementary DNA
CO_2	Carbon dioxide
CXCL9	Chemokine (C-X-C motif) ligand 9
CXCL10	Chemokine (C-X-C motif) ligand 10
CXCR3	Chemokine (C-X-C motif) receptor 3
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
FasL	Fas ligand
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HBSS	Hanks' Balanced Salt Solution
HIST1H3G	Histone cluster 1, H3g
ICAM-1	Intracellular adhesion molecule 1
ICOS-L	Inducible T cell costimulator ligand
IFI30	Interferon, gamma-inducible protein 30
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-γ
IL-1α	Interleukin 1α
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-15	Interleukin 15
IL-18	Interleukin 18
INK128	TORC1/2 inhibitor MLN0128
LFA-3	Lymphocyte function-associated antigen 3

M199	Medium 199
MHC	Major histocompatibility complex
MKI67	Marker of proliferation Ki-67
mTOR	Mechanistic target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NFκB	Nuclear factor of kappa light polypeptide gene enhancer in B cells
NK	Natural killer
OX40-L	OX40 ligand
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PD-L1	Programmed death-1 ligand 1
PD-L2	Programmed death-1 ligand 2
qPCR	Quantitative polymerase chain reaction
RAPTOR	Regulatory-associated protein of mTOR
RICTOR	RAPTOR-independent companion of TOR
RNA	Ribonucleic acid
SD	Standard deviation
SMC	Smooth muscle cell
SPP1	Secreted phosphoprotein 1
STAT1	Signal transducer and activator of transcription 1
T_H1	T helper 1
TLR3	Toll-like receptor 3
TNF	Tumor necrosis factor
TOP2A	Topoisomerase (DNA) II alpha 170kDa
VCAM-1	Vascular cell adhesion molecule 1
VSMC	Vascular smooth muscle cell

1 Introduction

1.1 Heart transplantation and graft rejection

Heart transplantation prolongs survival and improves the quality of life for patients with end-stage heart failure and has been the treatment of choice for these patients since the 1980s [1, 2]. The major barrier to successful organ transplantation is host immune responses that view the transplanted tissue as foreign and lead to destruction and rejection of the graft [3]. Acute rejection, characterized by leukocyte infiltration of the graft that leads to graft cell necrosis and/or graft vessel thrombosis, typically occurs 1-2 weeks after transplantation and is mediated primarily by a T cell response in the transplant recipient [4]. Chronic rejection primarily presents as chronic vascular rejection, which develops over months to years and is characterized by graft vessel stenosis due to a progressive immune-mediated host response to graft blood vessels, resulting in fibrosis of the graft parenchyma due to ischemia.

Short-term survival after heart transplantation has improved greatly from the early years of the procedure due to advances in immunosuppressive therapy and updated surgical procedures [5]. However, there has been little change in long-term survival, which is primarily limited by chronic rejection, malignancy, and side-effects of long-term immunosuppression [2]. Currently, 1 year survival after heart transplantation worldwide is 85%, 5 and 10 year survival conditional on 1 year survival are 86% and 68% respectively, and median survival is 11 years [6].

1.2 Cardiac allograft vasculopathy

1.2.1 Disease characteristics

The major cause of late graft failure in heart transplant recipients is cardiac allograft vasculopathy (CAV), a manifestation of chronic vascular rejection [7]. CAV is a specific form of arteriosclerotic disease that affects both the epicardial and intramyocardial vessels of the transplanted heart and stops at the suture line [5]. CAV affects 8% of recipients by year 1 after transplant, 30% by year 5, and 50% by year 10 [8]. CAV lesions are characterized by concentric intimal hyperplasia and inadequate outward vessel remodeling [9]. Early intimal lesions may be eccentric and focal but as the disease progresses and the lesions advance, the graft vessels become affected by extensive, confluent luminal stenosis. [7]. Accompanying vasoregulatory dysfunction that leads to impaired vasodilation and/or abnormal vasoconstriction also contributes to the reduced lumen size [4]. Progressive stenosis eventually leads to organ hypoperfusion and graft failure due to ischemia. All graft recipients show some degree of graft vascular remodeling but only a subset develop clinically significant chronic vascular rejection. However, patients with late graft failure almost always show extensive graft arterial and arteriolar lumen loss, in addition to fibrosis of the myocardium due to ischemia. Similar arteriosclerotic changes are seen in the graft vasculature of all other solid organ transplants [10].

The arterial vessel wall is divided into three compartments: the intima, media, and adventitia. In the healthy vessel wall, the intima consists of an endothelial cell (EC) monolayer on a basement membrane with a small amount of underlying smooth muscle cells (SMCs) and extracellular matrix

(ECM) and is separated from the media by the internal elastic lamina [11, 12]. The media consists primarily of SMCs and ECM and is separated from the adventitia by the external elastic lamina. Lastly, the adventitia is primarily composed of loose connective tissue but contains a small amount of nerve fibers, microvessels, and vascular stem cells. Each of these compartments can contain a small number of mononuclear leukocytes that can increase dramatically in number with inflammation.

The majority of the hyperplastic intima in CAV lesions is comprised of SMCs of donor origin and ECM thought to be produced by these cells [13, 14]. These SMCs could be medial cells that have migrated into the intima, resident intimal cells that have expanded in number, cells that have arisen from progenitor cells at the medial/adventitial border, cells that have arisen from endothelial-mesenchymal transition, or a combination of these possibilities [7]. Another prominent feature of the hyperplastic intima is an infiltrate of both CD4^{+} and CD8^{+} T cells and macrophages of host origin immediately beneath the luminal endothelium [13]. The intima also contains microvessels of graft origin and is covered by an intact EC lining [7]. The vessel wall compartments are not uniformly affected, as host T cells and macrophages predominantly infiltrate the intima and adventitia, while the media remains relatively spared [15].

CAV and conventional atherosclerosis have many similarities [16]. Both diseases are characterized by leukocyte infiltration, intimal SMC migration and ECM accumulation, endothelial dysfunction, increased expression of cell adhesion molecules, and similar cytokine profiles. The two diseases also have important differences such as in lesion distribution and organization.

1.2.2 Resident vascular cells in CAV pathogenesis

The precise pathogenesis of CAV remains unclear. CAV is considered to be primarily an alloimmune process, although nonimmune factors also contribute significantly to the pathogenesis [17]. The graft vasculature is targeted by the immune system through cytotoxic CD8^{+} T cells that induce vascular cell death, effector CD4^{+} T cells that produce various inflammatory cytokines, and graft-reactive antibodies produced by B cells [10]. This leads to vascular cell injury and alteration of graft vascular cell phenotypes, which eventually results in arteriosclerotic thickening and vasoregulatory dysfunction of allograft arteries. Other potential contributors to CAV lesion formation include conventional risk factors of atherosclerosis, pre- or peritransplant injuries, infection, and innate immunity [7]. Resident vascular cells, such as ECs and vascular SMCs (VSMCs), take an active part in immune-mediated inflammation and influence the adaptive immune response, and therefore are important participants in CAV pathogenesis [12].

ECs are present in the intima, where they form the inner lining of blood vessels, and the adventitia, where they line a number of microvessels. The intima and adventitia are commonly affected in immune-mediated disorders, in contrast to the media, which contains no ECs under normal circumstances [18]. ECs basally express both MHC class I and II molecules in situ and upregulate both types in response to inflammatory cytokines such as IFN-γ and TNF [10]. ECs from coronary arteries affected by CAV express significantly higher levels of MHC molecules than ECs from normal coronary arteries or coronary arteries affected by conventional atherosclerosis [13]. In culture, the expression of class I molecules decreases and that of class II molecules almost disappears, although

in both cases it can be restored by treatment with human IFN-γ [12]. ECs do not express B7 molecules required for costimulation of naive T cells, but express a variety of other positive costimulatory molecules that allow them to act as antigen presenting cells that can activate alloreactive memory T cells to proliferate and produce cytokines [10, 18]. These costimulatory molecules include LFA-3, ICAM-1, OX40-L, and ICOS-L [12, 18]. Human ECs also express negative costimulatory molecules that inhibit T cell activation such as PD-L1 and PD-L2. They are also capable of producing various inflammatory cytokines, such as IL-1α, IL-6, IL-15, and IL-18, which contribute to the activation and differentiation of T cells [19].

VSMCs are the most frequent cell type of larger vessel walls and the main component of the hyperplastic intima in CAV. They are also the principal cell type of the media and are therefore likely to be responsible for the medial immunoprivilege seen in CAV and other immune-mediated disorders [18]. Cultured VSMCs basally express MHC class I molecules and can be induced to express MHC class II molecules in response to IFN-γ, which also increases the expression of MHC class I molecules several folds. [20]. VSMCs do not express B7 molecules required for the activation of naive T cells and are also not capable of activating resting CD4^{+} T cells to proliferate and produce cytokines under the same experimental conditions that ECs can [18, 21]. However, they are capable of restimulating CD4^{+} T cells that have previously been activated by ECs, as is the case with T cells that must come into contact with ECs before infiltrating further into the vessel wall where VSMCs are present.

IFN-γ-treated VSMCs are also capable of reducing CD4^{+} T cell activation by ECs, most likely due to a combination of IFN-γ inducible production of indoleamine 2,3-dioxygenase (IDO) and either a lack of positive costimulatory molecule expression or expression of an unidentified negative costimulatory molecule [15]. IDO is the rate-limiting enzyme for tryptophan catabolism. Its upregulation, induced by IFN-γ, leads to tryptophan depletion, which inhibits allogeneic T cell activation, proliferation, and accumulation in vitro and in vivo. VSMCs express many of the same positive and negative costimulatory molecules as ECs, such as LFA-3, ICAM-1, PD-L1, and PD-L2 [18]. However, they do not basally express OX40-L or upregulate ICOS-L in response to proinflammatory cytokines like ECs. The difference in OX40-L expression seems to explain why ECs are capable of stimulating allogeneic memory T cell proliferation and cytokine production, but VSMCs are not. VSMCs are also capable of producing a variety of other proinflammatory molecules in response to microbial infection or cellular stress and injury, such as cytokines that can activate macrophages or influence T cell differentiation, adhesion molecules that facilitate leukocyte trafficking, and chemokines that can recruit macrophages or T cells [18].

Past research has suggested that IFN-γ is the primary mediator of CAV [7]. IFN-γ is produced by a variety of cell types, but the primary source is T helper type 1 (T$_H$1) cells, a subset of CD4[+] effector T cells [17]. T$_H$1 cytokines, such as IFN-γ, and associated chemokines have been shown to predominate in CAV lesions [22]. IFN-γ has various effects on resident vascular cells, including upregulation of MHC molecules in both ECs and VSMCs. MHC molecule upregulation in ECs enhances their activation of T cells, which increases the targeting of blood vessels by activated effector T cells [10]. IFN-γ also induces EC expression of chemokines that facilitate the migration of T cells into the vessel wall. Immunologic injury of ECs and SMCs can trigger or promote the development of transplant

arteriosclerosis and appears to be mediated primarily by cytotoxic CD8$^+$ T cells and inflammatory cytokines such as IFN-γ, which increases EC susceptibility to FasL-mediated cell death [10]. Lastly, IFN-γ has been shown to be an important mediator of intimal hyperplasia by stimulating the proliferation of VSMCs. [23].

1.2.3 Experimental models of CAV

Studies on CAV and its pathogenesis rely on a number of different experimental models. Currently, the experimental model that best mimics CAV is a humanized mouse model of artery allograft rejection [24]. Conventional mouse models have not proven suitable for studies on CAV in humans because of various immunological differences between the two species. In the humanized mouse model, human artery segments are transplanted into immunodeficient mouse hosts and allowed to quiesce for 30 days. The artery segments are then retransplanted into a second mouse host that has previously been inoculated with human PBMCs or purified T cells allogeneic to the artery segment. Over the next 21 days, the mouse develops lesions greatly resembling CAV lesions. Studies on CAV also rely on analysis of clinical specimens and cell and artery culture. Cell culture makes it possible to study only a single cell type at once, although the cells may lose some of their differentiated characteristics after removal from the in vivo environment and/or prolonged culture. This problem is avoided in artery organ culture where cells remain fully differentiated and the original organization of tissue is maintained, but samples contain many different cell types.

1.3 mTOR inhibition by rapamycin

1.3.1 mTOR inhibitors

Currently, the only treatment for established CAV is retransplantation [25]. However, mechanistic target of rapamycin (mTOR) inhibitors, such as rapamycin (sirolimus) and everolimus, have been shown to have a preventive effect if treatment is started early after transplantation, lowering the incidence and severity of CAV. Two clinical trials have examined the preventive effects of mTOR inhibitors. The first trial, conducted at 52 centers in Europe, North America, and South America, showed that treatment with everolimus, compared with azathioprine, resulted in significantly less intimal expansion of graft arteries and a lower incidence of CAV 12 months after transplantation [26]. The second trial, conducted at five centers in Australia and New Zealand, showed that treatment with rapamycin, compared with azathioprine, resulted in significantly less transplant coronary disease at 6 and 24 months [27]. A few studies have examined the effect of mTOR inhibitors on established CAV progression with contradictory results, although stronger evidence suggests that their use does not influence the progression of established disease [28-30]. This difference might reflect that early and late CAV development and progression are influenced by different pathophysiological mechanisms that would have to be targeted in different ways [28].

Rapamycin, the first mTOR inhibitor to be discovered, is a bacterially derived, anti-fungal macrolide that has proven to be a potent immunosuppressant [31]. Rapamycin was approved for use in kidney transplantations in 1999 after being shown to reduce the incidence of acute rejection episodes in combination with ciclosporin and corticosteroids in clinical trials [32]. Since then, its use has been

expanded to include extrarenal organ transplantation, including heart transplantation, where in addition to reducing the incidence of acute rejection, mTOR inhibitors also influence CAV development, as discussed above. Currently, rapamycin-eluting coronary stents are also commonly used in angioplasty, significantly reducing in-stent restenosis and the incidence of major adverse cardiac events following the procedure [33].

1.3.2 Mechanism of mTOR inhibition

mTOR is a serine/threonine protein kinase that is considered to be a central regulator of cell growth and immune responses [34]. mTOR signaling proceeds via two distinct complexes, mTOR complex 1 (mTORC1) and complex 2 (mTORC2). These complexes are comprised of mTOR and several other proteins. In particular, mTORC1 contains the regulatory-associated protein of mTOR (RAPTOR) and mTORC2 contains the scaffolding protein RAPTOR-independent companion of TOR (RICTOR). The effect of rapamycin on mTOR signaling is still not fully understood but it has been demonstrated that it affects mTORC1 and mTORC2 in different ways.

Rapamycin binds to the intracellular protein FK506-binding protein 12 (FKBP12) to generate a drug-receptor complex that can bind to the FKBP12 rapamycin-binding domain of mTOR [35]. When this binding occurs to mTOR as a part of mTORC1 it is thought to weaken the ability of RAPTOR to bind to mTOR, which results in an inhibition of mTORC1 kinase activity [36]. The FKBP12-rapamycin complex does not bind to mTORC2. However, the complex binds to free mTOR and can prevent the binding of newly synthesized mTOR to RICTOR [37]. This reduces the amount of mTORC2 that is assembled and in that way inhibits mTORC2 signaling. Because of these different mechanisms, mTORC1 inhibition takes place just minutes after treatment but mTORC2 inhibition only after prolonged treatment of at least 24 hours.

1.3.3 mTOR inhibition in vascular cells

Rapamycin inhibits or decreases the proliferation and function of T, B, and NK cells [38]. It also reduces the alloimmunogenicity of dendritic cells (DCs), the principal antigen presenting cell of the immune system. Rapamycin treatment impairs the generation and maturation of DCs and reduces their surface expression of MHC class II molecules and costimulatory molecules such as CD40, B7.1, and B7.2. It also impairs their ability to produce and secrete various inflammatory cytokines. Rapamycin-treated DCs weakly stimulate T cells and induce hyporesponsiveness and apoptosis in alloreactive T cells. However, they retain the ability to stimulate and expand regulatory T cells.

As mentioned earlier, graft ECs can also function as antigen presenting cells, and as such play an important role in CAV pathogenesis. Human ECs appear to primarily be capable of activating resting memory cells but not resting naive T cells [12]. Rapamycin treatment of ECs, through inhibition of both mTORC1 and mTORC2, alters the expression of various molecules that are important in CAV pathogenesis, such as PD-L1 and PD-L2, IL-6, VCAM-1, and various costimulatory molecules. Rapamycin pretreatment has been shown to reduce the alloimmunogenicity of ECs, which results in less proliferation and cytokine secretion by stimulated T cells, and T cell infiltration and intimal expansion of graft vessels in a humanized mouse model.

T cells stimulated by rapamycin-treated human umbilical vein ECs secrete less IL-2 and IFN-γ and proliferate less than T cells stimulated by control ECs [19]. Additionally, rapamycin pretreatment of grafts transplanted into a humanized mouse model results in a reduction in T cell infiltration into the vessel intima and intimal expansion, compared with control grafts. These effects have been linked to upregulation of the T cell-inhibitory molecules PD-L1 and PD-L2 on both cultured ECs and graft ECs of the humanized mouse model. Upregulation of PD-L1 and PD-L2 also appears after knockdown of mTOR and RAPTOR but not RICTOR, suggesting that this effect is primarily mediated by inhibition of mTORC1 signaling. Rapamycin pretreatment also inhibits secretion of the T cell-activating cytokine IL-6 by cultured ECs in response to TNF or IFN-γ, an effect that seems to be mediated by inhibition of both mTORC1 and mTORC2 signaling. However, this decrease in IL-6 secretion does not seem to be as important as the upregulation of PD-L1 and PD-L2 in the reduction of EC alloimmunogenicity.

The decrease in T cell infiltration and intimal expansion after rapamycin pretreatment of grafts transplanted into humanized mouse models could also be a result of mTORC2 inhibition. Rapamycin pretreatment has been shown to reduce the ability of TNF-treated human umbilical vein ECs to capture T cells under conditions of venular flow [39]. This effect has been linked to inhibition of TNF-induced VCAM-1 expression of the ECs, both in vitro and in vivo. Inhibition of VCAM-1 expression also appears after knockdown of mTOR and RICTOR, but not RAPTOR, suggesting that this effect is primarily mediated by inhibition of mTORC2 signaling.

The studies discussed above have focused on the role of ECs in CAV pathogenesis. In vivo rapamycin treatment of grafts transplanted into humanized mouse models in these studies is likely to also be affecting VSMCs, which could be contributing to the observed effect of the treatment. In addition to its immunosuppressive effects on ECs, rapamycin has been shown to inhibit VSMC proliferation both in vivo and in vitro [40]. Recently, a microarray analysis was performed on artery grafts explanted from immunodeficient mice (G. Tellides, unpublished work, 2015). Human coronary arteries were transplanted into immunodeficient mice that were then treated with mTOR inhibitors, either everolimus or INK128, for 24 hours prior to removal of the graft for the second transplantation. The second mouse hosts were sacrificed 6 hours after transplantation and the grafts harvested. Results for particular genes of interest are shown in Table 1. Everolimus and INK128 had a similar effect on gene expression. mTOR inhibition primarily downregulated two groups of genes, cell proliferation genes and inflammatory genes that are typically induced by IFN-γ and STAT1. In general, the changes detected by microarray were more consistent for the proliferation than the inflammation genes. From the group of over 20 proliferation-associated genes, we selected 4 for further study, including *HIST1H3G*, the most highly regulated gene in the microarray study, and *CCNA2*, *MKI67*, and *TOP2A*, which the laboratory has previously determined to be robust markers of VSMC proliferation in a murine model [41]. We also selected all 5 inflammatory genes identified by the microarray for further study, namely *CXCL9*, *CXCL10*, *IFI30*, *SPP1*, and *TLR3*. In addition, we measured the expression of the housekeeping gene *GAPDH* as well as the inflammatory gene *IL6*, which is known to be induced after artery transplantation, artery culture, and VSMC culture but was not altered in the microarray. The former was useful for expression normalization and the latter as a control for our experimental conditions. A brief description of the product and primary function of each gene is provided in Table 2.

Table 1. Results from microarray analysis of arteries from a humanized mouse model

Gene	Vehicle/mTOR inhibitors	P value
CXCL9	2.33	0.0211
CXCL10	6.09	0.0566
CCNA2	2.21	0.0050
HIST1H3G	5.79	0.0082
IFI30	1.93	0.0086
IL6	0.84	0.3379
MKI67	1.78	0.0129
SPP1	2.18	0.0088
TLR3	1.62	0.0006
TOP2A	2.50	0.0050

Table 2. Description of target gene products and primary functions[a]

Gene	Product	Primary functions
Cell proliferation genes		
CCNA2	Cyclin-A2	Activation of CDC2 or CDK2 kinases and promotion of cell cycle G1/S and G2/M transitions
HIST1H3G	Histone H3.1	Packaging of the chromosomal fiber into a nucleosome
MKI67	Antigen KI-67	Associated with and possibly necessary for cellular proliferation
TOP2A	DNA topoisomerase 2-alpha	Control and alteration of the topologic states of DNA during transcription
Inflammatory genes		
CXCL9	C-X-C motif chemokine 9	Chemotactic attraction of lymphocytes
CXCL10	C-X-C motif chemokine 10	Stimulation of monocytes, NK and T cell migration, and modulation of adhesion molecule expression
IFI30	Gamma-interferon-inducible lysosomal thiol reductase	Role in MHC class II-restricted antigen processing
IL6	Interleukin 6	Role in inflammation and B cell maturation
SPP1	Osteopontin	Upregulation of interferon-γ and interleukin 12
TLR3	Toll-like receptor 3	Recognition of dsRNA and induction of NFκB activation and type I interferon production

[a]Gene information was obtained from the NCBI RefSeq database[42]

2   Aim of this study

As previously discussed, resident vascular cells such as ECs and VSMCs are important participants in CAV pathogenesis. Few studies have examined the effects of mTOR inhibitors on VSMCs and little is known about their mechanism of action in this particular cell type. The aim of this study was to examine whether mTOR inhibition by rapamycin decreases basal and cytokine-induced transcripts for cell proliferation and inflammatory genes in cultured VSMCs and coronary arteries, and to compare the results with the microarray analysis previously performed on artery grafts explanted from a humanized mouse model.

3   Materials and methods

3.1   Arterial tissue

Human subject protocols were approved by the Yale Human Investigations Committee and the New England Organ Bank. Aortas and coronary arteries were obtained from the explanted hearts of 4 cadaveric organ donors. The arteries were denuded of adventitia and placed in cold saline on ice for transfer.

3.2   Artery culture

Human coronary arteries had previously been cut into 60-100 mg pieces and either placed immediately into liquid nitrogen followed by storage at -80°C or placed into wells of 6-well plates for culture. The arteries were cultured in M199 media (Gibco) supplemented with 10% FBS and treated with either vehicle (0.01% DMSO in HBSS) or rapamycin (Calbiochem) at 100 ng/ml for 6 or 72 hours. During treatment, the arteries were maintained in 5% CO_2 at 37°C. After treatment, the arteries were placed into liquid nitrogen followed by storage at -80°C.

3.3   Cell culture

Human aortic or coronary VSMCs were isolated by explant outgrowth and serially cultured in M199 media supplemented with 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 ug/ml streptomycin (all from Gibco). VSMCs at passage level 3 to 4 were placed in gelatin-coated wells of 6-well culture plates and maintained in 5% CO_2 at 37°C for 2-3 days. The cells were then either treated with vehicle or rapamycin at 1, 10, or 100 ng/ml for 6 hours, or with 100 ng/ml rapamycin for 24 or 72 hours. For the last 6 hours of each treatment period the cells were either treated with vehicle (0.2% BSA in DPBS) or TNF (Invitrogen) at 1 ng/ml.

3.4   Real-time quantitative polymerase chain reaction

Total RNA was extracted from arteries and cells using RNeasy Mini Kits (QIAGEN) with on-column DNase I treatment using RNase-Free DNase Sets (QIAGEN). Reverse transcription was performed using iSCRIPT™ cDNA Synthesis Kits (Bio-Rad). Quantitative real-time PCR was performed using TaqMan® Gene Expression Assays (*CXCL9*, *CXCL10*, *HIST1H3G*, *TOP2A*, *CCNA2*, *SPP1*, *IFI30*, *TLR3*, *MKI67*, *IL6*, *GAPDH*, and *ACTB*, all from Applied Biosystems), TaqMan® Universal PCR Master Mix (Applied Biosystems), and a C1000™ Thermal Cycler equipped with a CFX96™ Real-Time System (Bio-Rad). All cDNA samples were run in duplicate. Target gene expression was normalized to that of *GADPH* and trends additionally verified to expression of *ACTB*.

3.5   Statistical analysis

Student's t-test and one-way ANOVA were performed using the Prism software program (GraphPad Software). Differences with $p < 0.05$ were considered to indicate statistical significance.

4   Results

4.1   Rapamycin downregulates proliferation genes in VSMCs in vitro

To determine the effect of rapamycin on transcription of the cell proliferation genes *CCNA2*, *HIST1H3G*, *MKI67*, and *TOP2A* in cultured VSMCs, qPCR results from cells treated with both TNF and vehicle were analyzed together, as TNF did not have a significant effect on the transcription of these genes. No significant change in expression was found after treatment with 1, 10, or 100 ng/ml rapamycin for 6 hours. However, after 24 hours of treatment with 100 ng/ml rapamycin, significant downregulation was observed for *HIST1H3G* ($p < 0.01$), *CCNA2*, *MKI67*, and *TOP2A* ($p < 0.0001$). After 72 hours of treatment with 100 ng/ml rapamycin, significant downregulation was observed for *CCNA2*, *MKI67*, and *TOP2A* ($p < 0.01$) and the expression of *HIST1H3G* displayed a similar pattern without reaching statistical significance ($p = 0.0529$). The relative expression of these genes can be seen in Figure 1.
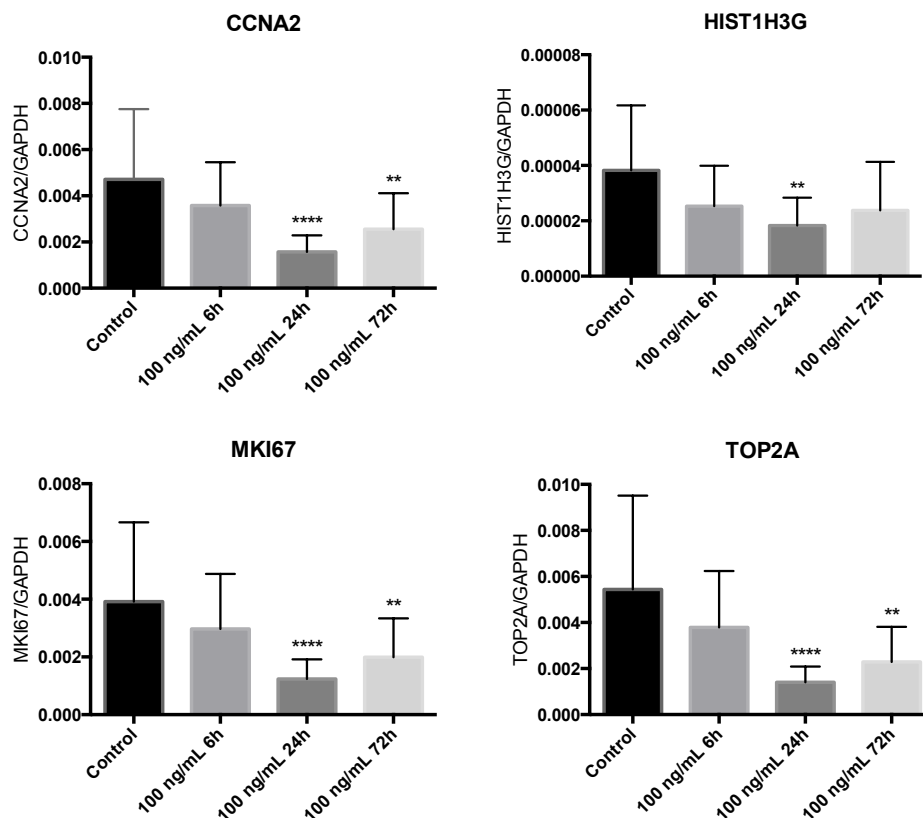

Figure 1. Expression of cell proliferation genes in cultured VSMCs
Significant downregulation was observed for *CCNA2*, *HIST1H3G*, *MKI67*, and *TOP2A* after 24 hours of treatment and for *CCNA2*, *MKI67*, and *TOP2A* after 72 hours of treatment. Target gene expression was determined by real-time qPCR and normalized to *GAPDH* expression. Results are expressed as mean with SD. **$p < 0.01$, ****$p < 0.0001$.

In cultured coronary arteries, no significant change in cell proliferation gene expression was found after treatment with 100 ng/ml rapamycin for 6 hours. However, significant downregulation was observed for *MKI67* and *TOP2A* ($p < 0.001$) after 72 hour treatment with 100 ng/ml rapamycin. The relative gene expression after treatment for 72 hours can be seen in Figure 2.
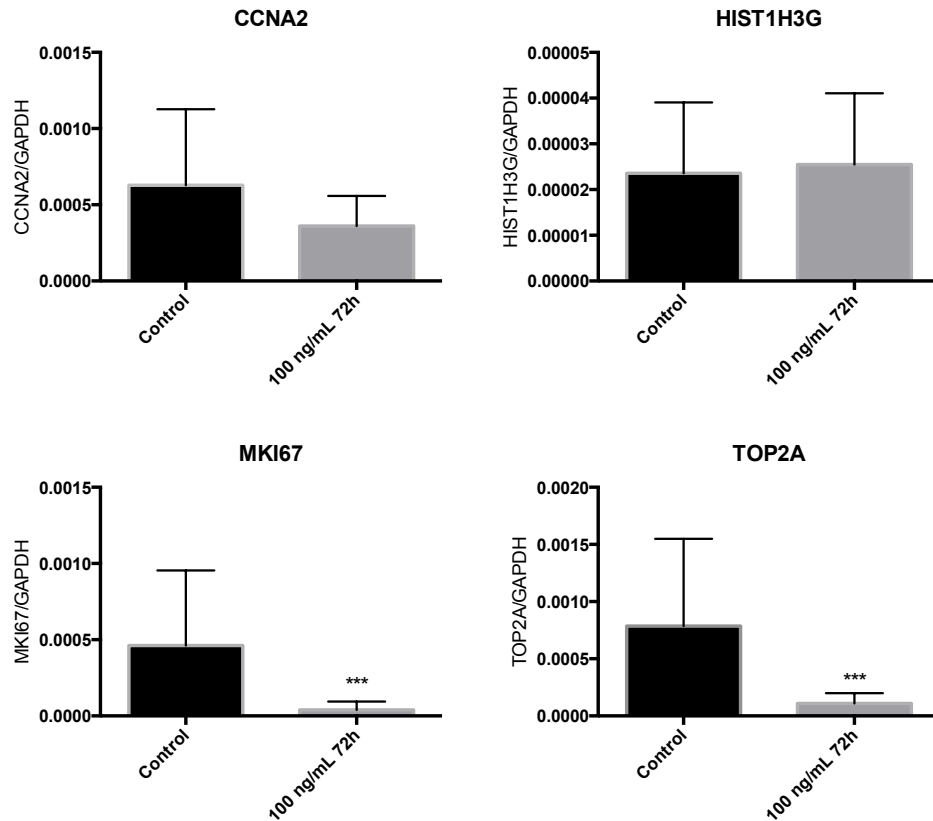
Figure 2. Expression of cell proliferation genes in cultured coronary arteries
Significant downregulation was observed for *MKI67* and *TOP2A* after treatment for 72 hours but not for *CCNA2* or *HIST1H3G*. Target gene expression was determined by real-time qPCR and normalized to *GAPDH* expression. Results are expressed as mean with SD.***$p < 0.001$.

4.2 Rapamycin weakly influences inflammatory gene expression in VSMCs in vitro

To determine the effect of rapamycin on transcription of the inflammatory genes *CXCL9*, *CXCL10*, *IFI30*, *SPP1*, and *TLR3* in cultured VSMCs, qPCR results from cells treated with TNF and vehicle were analyzed both separately and together, as TNF treatment resulted in upregulation of many of the inflammatory genes. In TNF-treated VSMCs, significant upregulation was observed for *CXCL9* ($p < 0.001$) and *CXCL10* ($p < 0.01$) after treatment with 100 ng/ml rapamycin for 72 hours. The relative expression of *CXCL9* and *CXCL10* in VSMCs can be seen in Figure 3. In both TNF- and vehicle-treated VSMCs, significant downregulation was observed for *TLR3* after treatment with 100 ng/ml rapamycin for 6 ($p < 0.01$), 24 ($p < 0.001$), and 72 hours ($p < 0.05$). No significant change in expression was found for other inflammatory genes. The relative expression of *IFI30*, *SPP1*, and *TLR3* in VSMCs can be seen in Figure 4A.
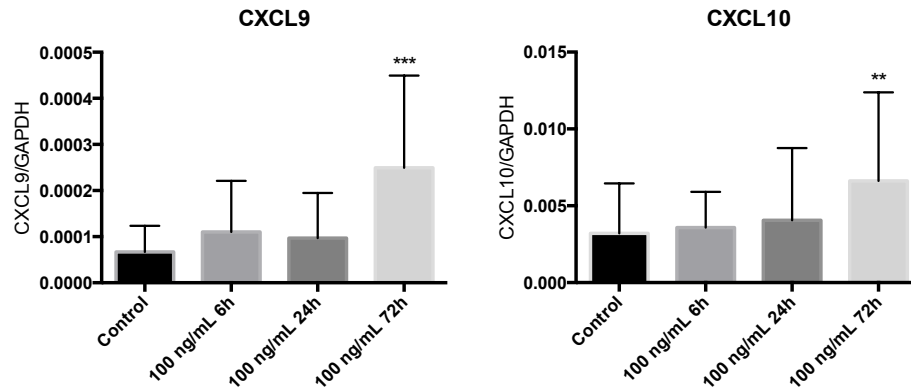
Figure 3. Expression of *CXCL9* and *CXCL10* in TNF-treated cultured VSMCs
Significant upregulation was observed for *CXCL9* and *CXCL10* after 72 hours of treatment. Target gene expression was determined by real-time qPCR and normalized to *GAPDH* expression. Results are expressed as mean with SD. **p<0.01, ***p<0.001.

In coronary arteries, no significant change in inflammatory gene expression was found after treatment with 100 ng/ml rapamycin for 6 hours. After 72 hours of treatment, significant downregulation was observed for *IFI30* (p<0.01) and *SPP1* (p<0.001), and the expression of *TLR3* displayed a similar pattern without reaching statistical significance (p=0.0596). The relative expression of *IFI30*, *SPP1*, and *TLR3* in coronary arteries can be seen in Figure 4B
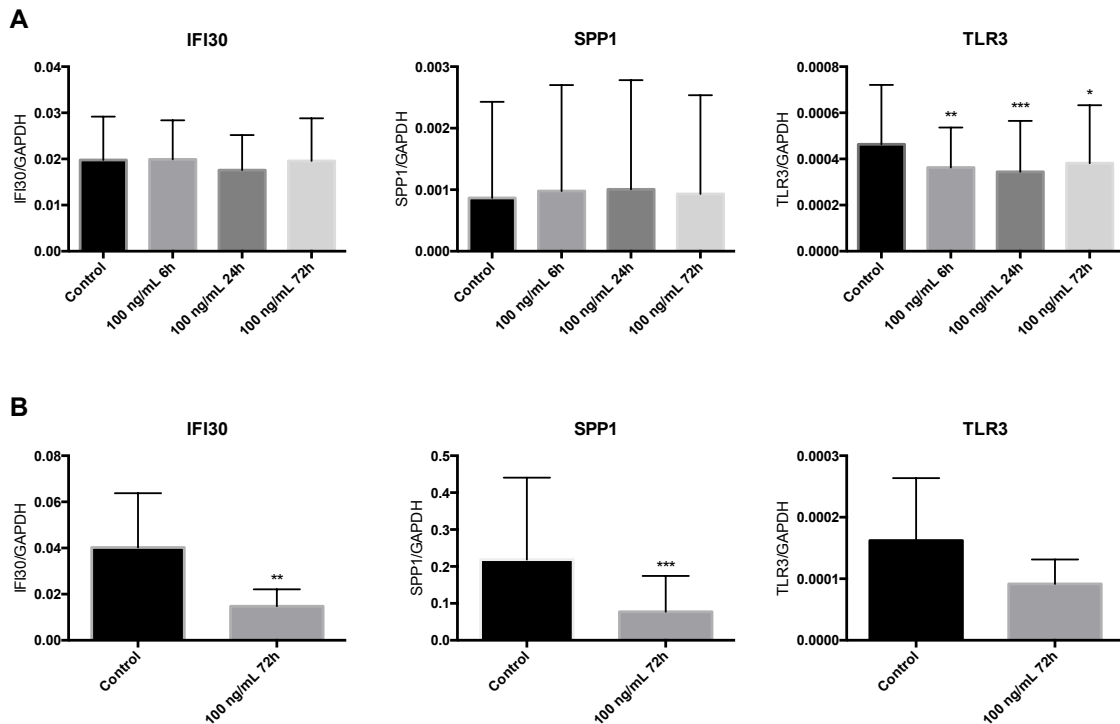
Figure 4. Inflammatory gene expression in cultured VSMCs and coronary arteries
(A) Significant downregulation was observed for *TLR3* in cultured VSMCs after 6, 24, and 72 hour treatment, but no change of expression was observed for *IFI30* and *SPP1*. (B) Significant downregulation was observed for *IFI30* and *SPP1* in cultured coronary arteries after 72 hour treatment, but the change in expression of *TLR3* did not reach statistical significance (p=0.0596). Target gene expression was determined by real-time qPCR and normalized to *GAPDH* expression. Results are expressed as mean with SD.*p<0.05, **p<0.01, ***p<0.001

5   Discussion

The aim of this study was to determine the transcriptional effects of mTOR inhibitors on VSMCs. A previously performed microarray analysis of human coronary arteries transplanted to immunodeficient mice and treated with mTOR inhibitors in vivo identified two major groups of proliferation- and inflammation-related genes that were downregulated by mTOR blockade. Several transcripts were selected for further investigation in model experimental systems of cultured coronary arteries and VSMCs.

In cultured VSMCs, the cell proliferation genes *CCNA2*, *HIST1H3G*, *MKI67*, and *TOP2A* were downregulated by rapamycin. Downregulation was most pronounced after 24 hours of treatment, suggesting that the optimal treatment duration for rapamycin lies somewhere between 6 and 72 hours. No difference in expression was found between groups treated with different concentrations of rapamycin for 6 hours, which could be because the treatment duration was too short for an effect to be observed. In cultured coronary arteries, *MKI67* and *TOP2A* were downregulated after 72 hours of rapamycin treatment, and *CCNA2* displayed a similar expression pattern without reaching statistical significance. However, no change in expression was observed for *HIST1H3G*. As the cultured coronary arteries were only treated for either 6 or 72 hours due to the small amount of samples available, we might have missed some of rapamycin's effects that would have been undetectable at the later time point. Additionally, even though proliferation gene expression in the coronary arteries was similar to that of the cultured VSMCs immediately after removal from the in vivo environment, after 72 hours it was up to 100 times lower in the arteries than in the cells, which might have resulted in difficulties in detecting a change in expression. The regulation of the cell proliferation genes that were examined in this study is consistent with the microarray analysis previously performed on coronary artery grafts from humanized mice, and suggest that in vitro models can be used to further study mechanisms of transcriptional control by mTOR inhibitors in VSMCs. The transcriptional effects of mTOR inhibition in VSMCs have not been studied before. Rapamycin is known to inhibit VSMC proliferation and the results of this study describe potential mechanisms underlying this effect [40]. This effect is also likely to be contributing to the reduction in intimal hyperplasia seen after mTOR inhibition in vivo, in addition to the previously reported reduction in EC alloimmunogenicity [19].

Several inflammation-related genes were downregulated in the microarray analysis, although the observed effect was weaker than for the cell proliferation genes. Of these genes, *CXCL9*, *CXCL10*, *IFI30*, *SPP1*, and *TLR3* were chosen to be examined further in this study, as *CXCL9* and *CXCL10* are known to be important in allograft rejection, and *IFI30*, *SPP1*, and *TLR3* have each been associated with conventional atherosclerosis in some way, although whether they take an active part in the pathogenesis is uncertain [43, 44]. In cultured VSMCs, only *TLR3* was significantly downregulated, with a peak effect after 24 hours of treatment. In cultured coronary arteries, significant downregulation was observed for the inflammatory genes *IFI30* and *SPP1* after rapamycin treatment for 72 hours, and a similar trend was observed for *TLR3* without reaching statistical significance.

The inflammatory chemokines *CXCL9* and *CXCL10* were downregulated in human coronary arteries after mTOR inhibitor treatment in vivo according to the microarray analysis, but no change in

expression was seen in cultured coronary arteries. However, upregulation of these chemokines was observed in TNF-treated VSMCs, with a peak effect after 72 hours of treatment. CXCL9 and CXCL10 are highly expressed in allograft rejection and, by binding to their receptor CXCR3, promote T cell activation, recruitment and allograft rejection [45]. A possible explanation for the different effect seen in cultured VSMCs compared to that seen in vivo is de-differentiation of the cells in culture. However, upregulation of *CXCL9* and *CXCL10* after rapamycin treatment has also been described in kidney tissue from a mouse model of ischemia-reperfusion injury after kidney transplantation [46]. In that particular model, the upregulation is believed to have a protective effect by recruiting natural killer T cells to the site of injury. The reason for this inconsistency between different in vivo experimental models is unclear, but possible explanations are differences between organisms or responses of kidney parenchymal cells and vascular cells.

Some of the strengths of this study are the use of human arteries and cells, that they were derived from several different donors, and the relevance of the cell type used for the disease of interest. The limitations of this study include that the artery culture experiments did not contain the time point that proved to be most effective when using the VSMCs, and that only a limited number of genes could be selected from the multiple genes that were identified as mTOR-dependent in the microarray analysis of in vivo coronary arteries.

In conclusion, the results of this study show that the regulation of several cell proliferation genes by mTOR in cultured arteries and VSMCs is consistent with the microarray analysis of coronary artery grafts in humanized mice, and suggests that these in vitro models can be used to further study mechanisms of transcriptional control by mTOR inhibitors in VSMCs. The most effective treatment duration for rapamycin in VSMCs could be determined further and the effect of different concentrations of rapamycin studied at that time point. The mechanism by which mTOR activates proliferation gene transcription is unknown, and would be of interest to study further. The weak and inconsistent effects observed on inflammatory gene expression in vitro may indicate false positive results of the microarray analysis, although an alternative explanation is that de-differentiation of cultured arteries and VSMCs is not suitable for this aspect of mTOR biology and that further in vivo studies are required.

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