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Rapamycin Effects Transcriptional Programs in Smooth Muscle Cells Controlling Proliferative and Inflammatory Properties

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ABSTRACT

Neointima formation, the leading cause of restenosis, is caused by proliferation of coronary artery smooth muscle cells (CASMCs) and is associated with infiltration by monocytes. Rapamycin inhibits neointima formation after stent implantation in humans. It reduces proliferation by its effects on mammalian target of rapamycin (mTOR) kinase. In this study, we investigated the expression of mTOR in human neointima and the effect of rapamycin on global transcriptional events controlling CASMC phenotype. In neointimal CASMCs, mTOR exhibited increased phosphorylation and was translocated to the nucleus compared with control. Comparative gene expression analysis of CASMCs treated with rapamycin (100 ng/ml) revealed down-regulation of the transcription factor E2F-1, a key regulator of G_1/S-phase entry, and of various retinoblastoma protein/E2F-1–regulated genes. In addition, we found changes in the expression of genes associated with replication, apoptosis, and extracellular matrix formation. Furthermore, rapamycin decreased the gene expression of endothelial monocyte-activating polypeptide-II (EMAP-II). This decrease of EMAP-II expression was reflected in a reduced adhesiveness of CASMCs for monocytic cells. Addition of EMAP-II counteracted the antiadhesive effect of rapamycin. Therefore, EMAP-II may comprise a mechanism of rapamycin-mediated reduction of the proinflammatory activation of CASMCs. The effects reported here of rapamycin on the down-regulation of genes involved in cell cycle progression, apoptosis, proliferation, and extracellular matrix formation in CASMCs provide an explanation of how rapamycin reduces CASMC proliferation. In addition, rapamycin may contribute to a reduction of inflammatory responses by reducing the adhesiveness of CASMC, a mechanism suggested to be mediated by the production and release of EMAP II.

Restenosis is the most important limitation of percutaneous angioplasty procedures. Although stent implantation reduces the risk of restenosis compared with other percutaneous treatment modalities, angiographic restenosis rates remain around 30% (Mintz et al., 1996; Kastrati et al., 1999). The late lumen loss after stent implantation is mainly caused by neointima formation as a result of tissue proliferation (Mintz et al., 1996). Rapamycin-coated stents have been shown to dramatically decrease the risk of in-stent restenosis in humans (Sousa et al., 2001). In several animal models of restenosis, rapamycin inhibits the proliferative response, causing neointima formation by enhancement of the level of $p27^{kip1}$ protein and activation of retinoblastoma protein (pRb) (Gallo et al., 1999; Poon et al., 2002). Nevertheless, rapamycin also inhibits neointima formation in $p27^{kip1}$ knockout mice via $p27^{kip1}$-independent mechanisms (Roque et al., 2001).

Rapamycin, an immunosuppressive, binds to cytosolic FKBP-12, and this complex inhibits the protein kinase mTOR (Gingras et al., 2001). The mTOR kinase is essential for viability and regulates translation initiation and cell cycle progression by altering the phosphorylation state of downstream targets such as the p70 S6 kinase (p70S6K) (Gingras et al., 2001). In T lymphocytes, inhibition of mTOR by rapamycin leads to inactivation of p70S6K and inhibits hyperphosphorylation of the tumor suppressor pRb, which is re-

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ABBREVIATIONS: pRb, retinoblastoma protein; CASMC, coronary artery smooth muscle cell; ECM, extracellular matrix; EMAP-II, endothelial monocyte-activating polypeptide-II; FKBP-12, FK506 binding protein 12; PI(3)K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; SMGM, smooth muscle growth medium-2; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; PCR, polymerase chain reaction; zVAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; zASTD-fmk, *N*-benzyloxycarbonyl-Ala-Ser-Thr-Asp- fluoromethylketone; YY1, yin&yang 1; CDK, cyclin-dependent kinase; p70S6K, p70 S6 kinase.

quired for the release of the transcription factor E2F (Brennan et al., 1999). The p70S6K is an important regulator of cell cycle progression in response to mitogens such as platelet-derived growth factor (Scott et al., 1996).

We have previously demonstrated that FKBP-12 is upregulated in coronary artery smooth muscle cells (CASMCs) from human in-stent neointima (Zohlnhöfer et al., 2001a,b), arguing for a role of FKBP-12 in signal transduction during neointima formation. However, a systematic analysis of the effect of rapamycin on CASMCs has not yet been performed.

Recently, the extent of inflammation has been shown to have a strong impact on restenosis. It has been demonstrated that medial injury significantly correlated with the degree of neointimal thickness and that the neointimal area occupied by macrophages significantly correlated with restenosis as well as the total number of inflammatory cells (Farb et al., 2002).

Thus, neointima formation may be mediated by inflammatory mechanisms explored in other models of vascular injury. In a model of ischemia-reperfusion, inflammation was prevented through inhibition of apoptosis associated with a reduced activation of endothelial monocyte-activating polypeptide-II (EMAP-II) (Daemen et al., 1999). EMAP-II is a proinflammatory cytokine and a chemoattractant for monocytes. In mouse embryo, EMAP-II mRNA was most abundantly expressed at sites of tissue remodeling in the presence of many apoptotic cells (Knies et al., 1998). The removal of dead cells required macrophages, which colocalize with areas of EMAP-II mRNA expression and programmed cell death (Knies et al., 1998). Active EMAP-II is a 23-kDa protein resulting from the cleavage of the intracellular 43-kDa pro-EMAP protein. In apoptotic cells, caspase-7–mediated cleavage and release of active EMAP-II is reported, suggesting that the coordinated program of cell death comprises the activation of an inflammatory reaction (Knies et al., 1998; Behrensdorf et al., 2000). The EMAP-II cytokine is released from the mammalian multisynthetase complex after cleavage of its p43/pro-EMAP-II component (Shalak et al., 2001). In our study, we investigated the regulation of mTOR kinase in human neointima and the effect of rapamycin on global gene expression patterns as well as the functional consequences with respect to proliferation, apoptosis, and CASMC adhesiveness.

Materials and Methods

Patients and Sample Preparation. Immunohistochemistry was performed on neointima from coronary in-stent restenosis (n = 2), restenotic peripheral arteries (n = 3), and healthy control media from coronary (n = 2) and peripheral arteries (n = 3). The investigation conformed to the principles outlined in the Declaration of Helsinki.

Cell Culture. Primary human CASMCs (CellSystems Biotechnologie Vertrieb GmbH, St. Katharinen, Germany) were cultured in smooth muscle growth medium-2 (SMGM-2; CellSystems) (37°C, 5% CO_2, 90% humidity). Cells were used between passages 4 and 7. CASMCs were grown to 40% confluence and afterward cultured in the presence or absence of 100 ng/ml rapamycin (Calbiochem, San Diego, CA) for the times indicated. For dose-response experiments, CASMCs were cultured in the presence or absence of 5, 20, 100, and 200 ng/ml rapamycin for 48 h. Cells were harvested at the end of the experiment. For mRNA preparation, CASMCs were washed twice, and 1×10^4 cells were lysed in 1 ml of lysis/binding buffer (Dynal Biotech, Lake Success, NY).

For induction of apoptosis, CASMCs were incubated at 37°C for 1 h in Hanks' balanced salt solution containing 1000 μM H_2O_2 and 100 μM ferrous sulfate. Subsequently, cells were cultured in SMGM-2 for 6 h for the annexin-V staining and for 12 h for the TUNEL assay. Mono Mac 6 cells, a human cell line with characteristics of mature monocytes (Ziegler-Heitbrock et al., 1988), were cultured in very low endotoxin-RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (Cambrex Bio Science Walkersville, Walkersville, MD).

Isolation of mRNA and Global Reverse Transcription-PCR. mRNA isolation, cDNA synthesis, and PCR amplification were performed as described previously (Zohlnhöfer et al., 2001a). Five independent experiments were performed for proliferating CASMCs and each time point of rapamycin treatment (24, 48, and 72 h).

Labeling of cDNA Probes and Hybridization to cDNA Arrays. Aliquots of 25 ng of each cDNA were labeled with digoxigenin-11-dUTP (Roche Applied Science, Indianapolis, IN) during PCR, and each probe was hybridized to three arrays (Atlas human cancer 1.2, human 1.2, and cardiovascular arrays; BD Biosciences Clontech, Palo Alto, CA) as described previously (Zohlnhöfer et al., 2001a). Detection of filter-bound probes was performed by the digoxigenin detection system (Roche Applied Science).

Developed films were scanned and analyzed using the Array Vision software (Imaging Research Inc., St. Catherines, ON, Canada). Background was subtracted, and signals were normalized to nine housekeeping genes present on each filter whereby the average signal of the housekeeping genes was set to 1 and the background to 0.

A selection of rapamycin-regulated genes was confirmed by gene-specific PCR. RNA (0.3 μg total) was reverse transcribed in a 20-μl reaction containing 1 μl of $Oligo(dT)_{12\text{-}18}$ (Roche Applied Science), 1 μl of 10 mM dNTPs, 4 μl of 5× First-Strand buffer, 2 μl of 0.1 M dithiothreitol, and 100 U of SuperScriptII (Invitrogen, Carlsbad, CA) at 44°C for 45 min. Afterward, PCR was performed using 0.75 ng of each cDNA in a 25-μl reaction containing PCR buffer (Sigma-Aldrich, St. Louis, MO), 200 μM dNTPs, 0.1 μM of each primer, and 0.75 U of *Taq* polymerase (Sigma-Aldrich). PCR products were subjected to electrophoresis on a 2% agarose gel containing ethidium bromide (1 μg/ml). Intensity of PCR signals was analyzed by means of densitometry using Array Vision software. Expression levels are shown in relation to β-actin expression, which was set at a value of 1.

Real-Time PCR. Total RNA was extracted from cells using the RNeasy mini kit (QIAGEN GmbH, Hilden, Germany). RNA was transcribed to cDNA using omniscript reverse transcriptase (QIAGEN GmbH) and random hexamers (Invitrogen, Karlsruhe, Germany). Real-time PCR was performed by the SybrGreen-PCR core reagents kit according to the manufacturer's instructions (Applied Biosystems, Darmstadt, Germany) using exon 6/7-spanning primers for E2F-1 (5′-CCCATCCCAGGAGGTCACTT-3′, 3′-GGACAACAGCGGTTCTTGCT-5′). Primer sequences for GAPDH were 5′-GAAGGTGAAGGTCG-GAGTC-3′ and 3′-GAAGATGGTGATGGGATTTC-3′.

Flow Cytometric Analysis of Apoptotic Cell Death. CASMCs were labeled with annexin-V and propidium iodide (Roche Applied Science). Ten thousand events per sample were analyzed by a FacsScan flow cytometer (BD Biosciences, San Jose, CA). Apoptotic cells were assessed by binding of annexin-V along with propidium iodide exclusion to demonstrate the integrity of the cell membrane that remains intact during apoptosis.

TUNEL Assay for Identification of Apoptotic Cells. CASMCs were labeled with TUNEL substrate (Roche Applied Science) according to the manufacturer's protocol. Five thousand events per sample were analyzed by a FacsScan flow cytometer.

Flow Cytometric Analysis of Caspase-3 Activity. CASMCs were stained with a phosphatidylethanolamine-conjugated antibody against active caspase-3 (BD Biosciences) according to the manufacturer's protocol. Five thousand events per sample were analyzed by a FacsScan flow cytometer.

Adhesion Assay. CASMCs were seeded onto 24-well plates (Costar, Cambridge, MA) in SMGM-2 in the presence or absence of

rapamycin (100 ng/ml) or the caspase inhibitor zVAD-fmk (50 μM) for 24 h. Afterward, CASMCs were incubated in the presence or absence of 40 μg/ml EMAP-II protein for 2 h. Another caspase inhibitor, ZASTD-fmk (50 μM), was also added 2 h before coincubation with Mono Mac 6 cells. Mono Mac 6 cells were washed twice in serum-free RPMI 1640 medium/HEPES 25 mM and coincubated (7×10^5cells/ml) with the prewashed CASMCs for 30 min. As a positive control, Mono Mac 6 cells were stimulated with EMAP-II protein (40 μg/ml) 2 h before coincubation. In another set of experiments, adhesiveness of CASMCs was assessed 6 h after induction of apoptosis with 100 μM H_2O_2 and 100 μM ferrous sulfate in the presence or absence of rapamycin (100 ng/ml) or the caspase inhibitor zVAD-fmk (50 μM) for 24 h. The plates were washed up to five times, and remaining adherent monocytic cells were quantified by counting 16 high-power fields using light microscopy.

Immunohistochemistry. For histology and immunohistochemistry, atherectomy specimens were fixed in 4% formaldehyde, pH 7.0, and embedded in paraffin. Serial paraffin sections (3-μm) were deparaffinized, dehydrated, and for antigen retrieval, pressure-cooked for 4 min in citrate buffer (10 mM, pH 6.0), followed by blocking of endogenous peroxidase (1% H_2O_2/methanol; 15 min) and preincubation with 4% dried skim milk in antibody diluent (DakoCytomation California, Inc., Carpinteria, CA). Immunostaining employed the streptavidin-alkaline phosphatase technique for α-actin and the streptavidin–horseradish-peroxidase technique (DakoCytomation ChemMate detection kit) for CD3 and mTOR. Primary antibodies against α-actin (DakoCytomation, 1:300), CD3 (DakoCytomation; 1:80), and mTOR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:30) and phosphorylated p*mTOR (Cell Signaling Technology Inc., Beverly, MA), which detects mTOR only when phosphorylated on Ser 2448, were used.

Statistical Analysis. Results of the gene expression analysis are reported as median expression values of five samples of each group. Differences between the groups were analyzed by analysis of variance using the general linear model (SPSS 9.0; SPSS Inc., Chicago, IL). A descriptive p value <0.025 was regarded as relevant; subsequently, genes with a minimum ratio of 2.5 and a minimal difference of 0.1 within their group medians were considered as differentially expressed. Hierarchical clustering of average linkage clustering with the centered correlation metric was used (Gene Cluster/Treeview; Eisen et al., 1998). Results of the experimental studies are reported as mean ± S.E.M. and were compared by paired t test. $P < 0.05$ was regarded as significant.

Results

Expression of mTOR in CASMCs of Nonatherosclerotic Arteries and Neointimal Lesions. As depicted in Fig. 1, the mTOR protein was detectable in endothelial cells (open arrow) and CASMCs (arrow) in the media from nonatherosclerotic arteries (Fig. 1A, brown) and in neointimal CASMCs (arrowhead) and T lymphocytes (open arrowhead) from restenotic arteries (Fig. 1B, brown). mTOR was found translocated into nuclei and exhibited increased phosphorylation in CASMCs (Fig. 1D, arrow) and T lymphocytes (Fig. 1D, open arrow) from human neointima compared with CASMCs from control specimens, arguing for an activation of mTOR during neointima formation.

Effect of Rapamycin on the Transcriptome of CASMCs. Using gene expression profiling of CASMCs treated with rapamycin for 24, 48, and 72 h, we identified 227 genes of 2231 (9.8%) that were differentially expressed compared with nontreated CASMCs (Figs. 2 and 3). In addition to the statistical analysis, the validity of expression data was supported by a substantial number of hybridization signals that were determined in duplicate in independent hybridization experiments with different arrays, which all showed a high degree of reproducibility. Nine examples of duplicate determinations are shown in Fig. 3 (marked with "{").

Hierarchical clustering of the differentially expressed genes demonstrated the down-regulation of the majority of the genes by rapamycin (Fig. 2). The sample dendrogram comprised three major branches. The first branch contained 62 genes that were already down-regulated by rapamycin after 24 h, the second branch included the 50 genes that were down-regulated gradually after 48 h, and the third branch included two genes that were up-regulated by rapamycin. Those of the first branch included many genes encoding for proteins of cell cycle regulation, replication, and apoptosis, whereas a number of genes of the second branch were associated with the regulation of apoptosis, extracellular matrix, and adhesion (Fig. 2, B and C). To gain a better insight into the effect of rapamycin on CASMC phenotype, we arranged the differentially expressed genes into functional clusters (Fig. 3).

As demonstrated in Fig. 3A, rapamycin led to down-regulation of 20 genes associated with DNA replication and the

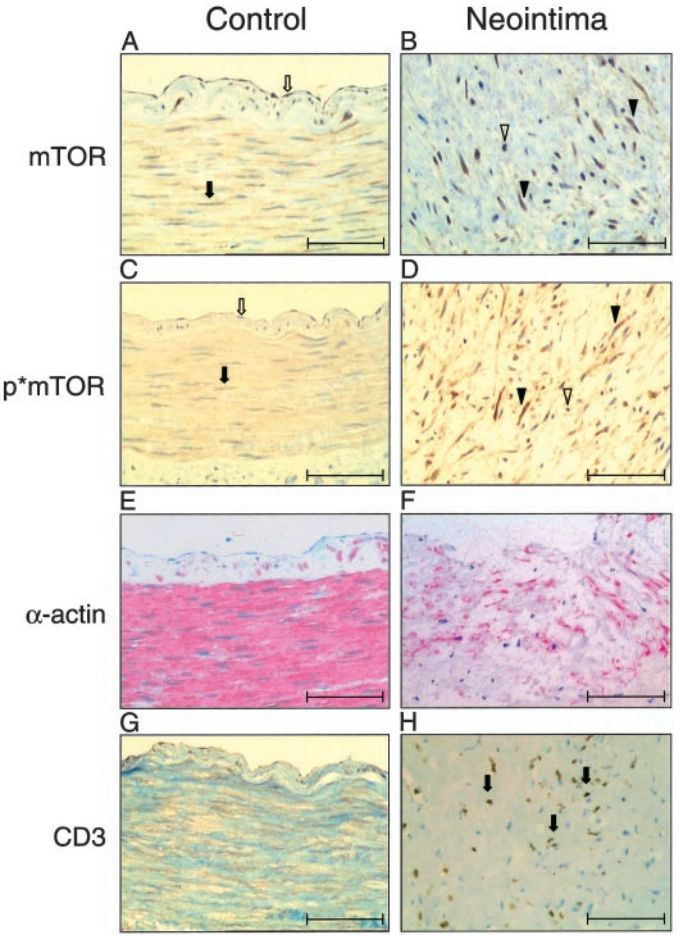

Fig. 1. Expression of mTOR protein. Immunohistochemical detection of mTOR (A, B) and phosphorylated p*mTOR (C, D) in endothelial cells (open arrow) and CASMCs (arrow) in nonatherosclerotic media (A, C; brown) and in CASMCs (arrowhead) and T lymphocytes (open arrowheads) of neointima from the arteria femoralis (B, D; brown). Detection of α-actin–positive CASMCs (E, F; red) in neointima and control media are shown, whereas CD3-positive T lymphocytes were only found in neointima (H; brown). The result shown is a representative of five experiments. Scale bars, 50 μm.

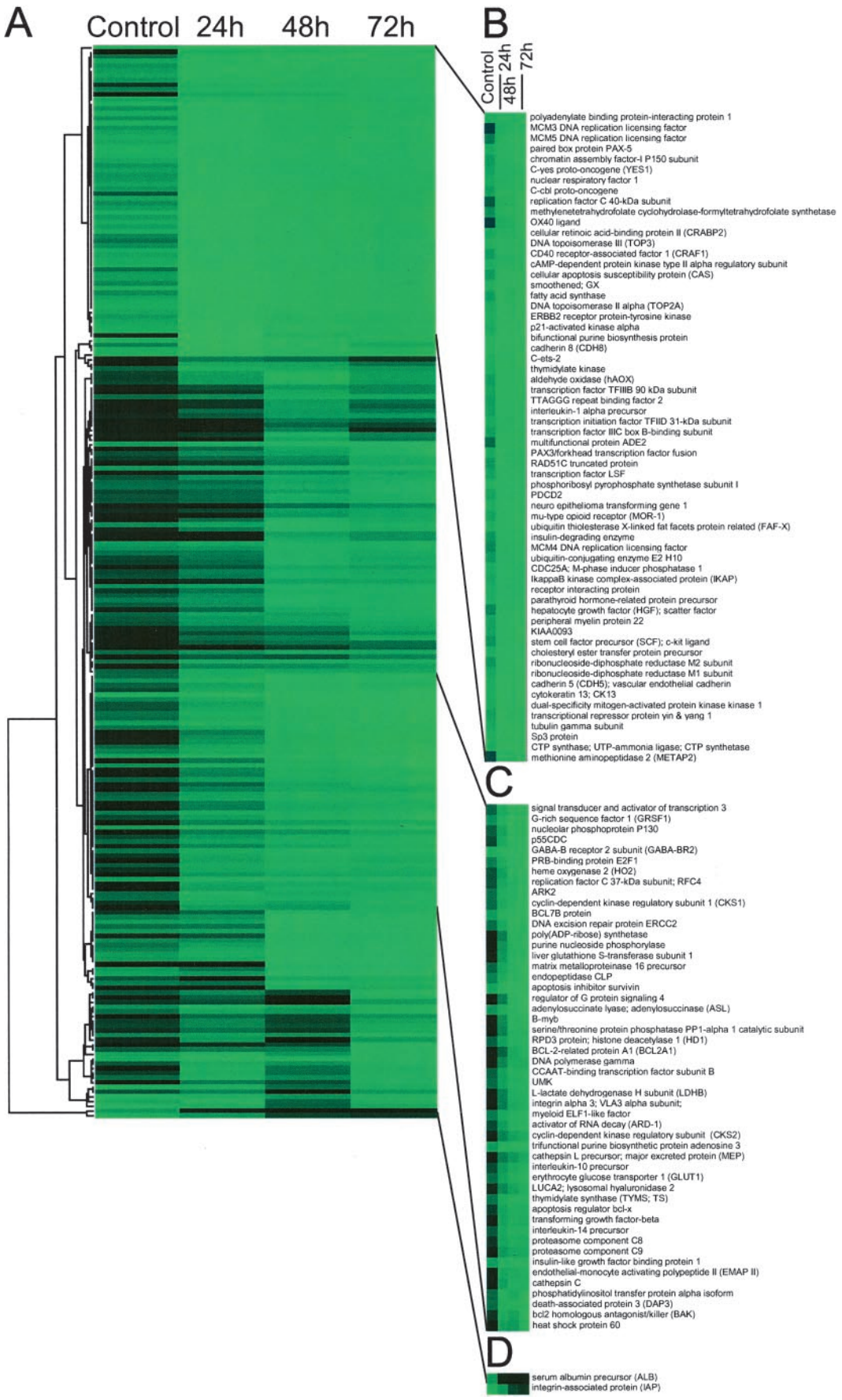

Fig. 2. Hierarchical cluster analysis of data from time course of proliferating CASMCs treated with rapamycin for 24, 48, and 72 h. A, genes were selected for this analysis if their expression level deviated by at least a factor of 2.5 among the groups and reached a descriptive p value <0.025. Each gene is represented by a single row, and each time point is represented by a single column. For each expression value, the median of the mRNA level of five experiments normalized to the mRNA expression level of the housekeeping genes is represented by a green value; color intensity is according to the expression level. B, amplified gene cluster showing 62 genes down-regulated after 24 h. C, amplified gene cluster showing 50 genes down-regulated after 48 h. D, amplified gene cluster showing the two consistently up-regulated genes.

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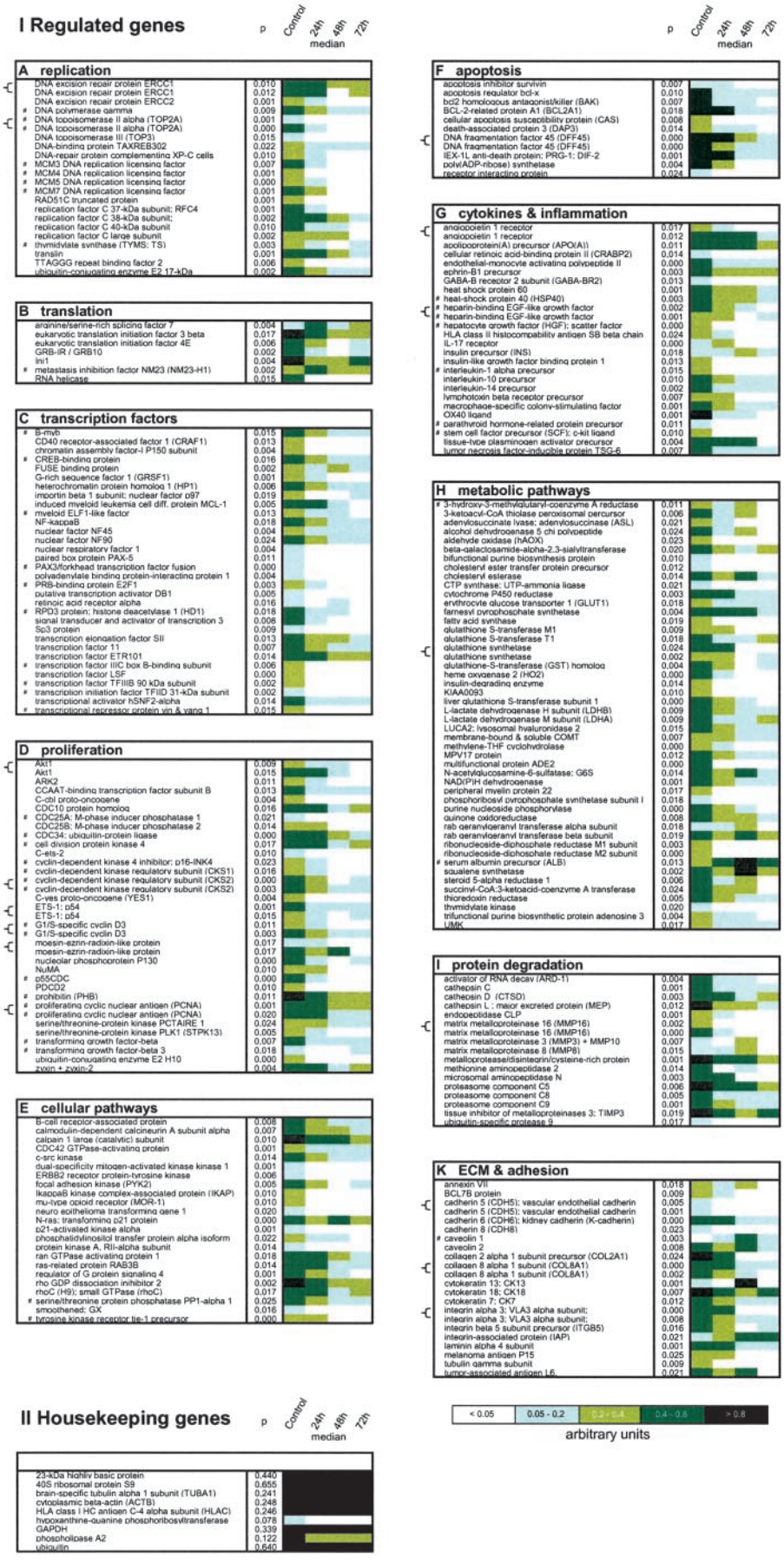

Fig. 3. Transcription profile of rapamycin-treated CASMCs. Cluster image showing mRNA levels of 227 genes upon 24-, 48-, or 72-h rapamycin treatment. Genes were clustered into 10 functional groups and the housekeeping genes. The expression pattern of each gene is displayed as a horizontal strip. Each column represents a single time point. For each expression value, the median of the mRNA level of five experiments normalized to the mRNA expression level of the housekeeping genes is represented by a green value according to the signal intensity scale at the bottom. #, genes associated with E2F-1/pRb signaling.

basal transcription machinery, such as the replication factor C-40. Thirty-two of the rapamycin-regulated genes encode transcriptional regulators (Fig. 3C), such as myeloid cell leukemia-1 and nuclear factor-κB. Likewise, rapamycin down-regulated the expression of the E2F-1 transcription factor. Simultaneously, we found repression of 41 genes associated with the E2F-1 signal transduction pathway. The cluster of pRb/E2F-1–regulated genes contained transcription factors including the transcriptional repressor protein yin&yang 1 (YY1) as well as the minichromosome maintenance DNA replication licensing factors. Furthermore, target genes such as cyclin-D3 and CDK4, which play important roles in cell cycle progression, showed a decrease in gene expression (Fig. 3).

Profound changes also took place in genes associated with apoptosis (Fig. 3F). We detected repression of genes encoding proteins with antiapoptotic function, e.g., Bcl-1–related protein A1, as well as genes of proapoptotic proteins, e.g., the cellular apoptosis susceptibility gene.

As presented in Fig. 3K, rapamycin inhibited the expression of functional important adhesion molecules and ECM proteins such as cadherin 5 and collagen type VIII α1. Likewise, genes involved in ECM degradation, such as matrix metalloproteinase 16, were down-regulated. Some changes took place in genes associated with leukocyte migration and adhesion, including EMAP-II, a chemoattractant for monocytes (Kao et al., 1994).

For further validation of hybridization signals and analysis of the dose response of CASMCs to rapamycin, we performed gene-specific PCR. We selected six genes with potential relevance to the therapeutic effect of rapamycin on neointima formation. The effect of rapamycin on gene expression was dose-dependent and already obvious at concentrations between 5 and 20 ng/ml (Fig. 4A). Furthermore, we performed gene-specific PCR for different genes associated with the regulation of apoptosis. As shown in Fig. 4B, rapamycin leads to sustained down-regulation of the mRNA for survivin, the cellular apoptosis susceptibility protein, and bcl2 homologous antagonist/killer. The survivin pathway interfaces with both the cell-death machinery and mechanisms of cell-cycle progression (Altieri, 2003), whereas bcl2 homologous antagonist/killer plays an important role in proapoptotic pathways (Cartron et al., 2003).

E2F-1 represents a central transcription factor, which is involved in the gene expression of many genes comprising the E2F-1–associated gene expression pattern. We, therefore, further corroborate the data regarding the effect of rapamycin on gene expression of E2F-1. Employing real-time reverse transcription-PCR, we were able to affirm the data of our gene expression analysis by cDNA arrays. As shown in Fig. 4C, incubation of CASMCs with rapamycin leads to a 50 to 60% reduction in E2F-1 mRNA expression after 24 to 72 h. Likewise, rapamycin significantly reduced cell proliferation of CASMCs grown in proliferation medium, in accordance with the literature (Poon et al., 2002) (data not shown).

Rapamycin Attenuates Apoptosis of CASMCs. As we identified major differences in the expression of genes associated with apoptosis in rapamycin-treated CASMCs (Figs. 3F and 4B), we functionally analyzed the effect of rapamycin on apoptosis. As shown in Fig. 5, A and B, rapamycin significantly reduced the basal rate of apoptosis compared with nontreated CASMCs. In addition, rapamycin treatment significantly attenuated H_2O_2-induced apoptosis compared with nontreated CASMCs, as detected by annexin-V staining (Fig. 5A) and a TUNEL assay (Fig. 5B).

To find clues as to how rapamycin prevents apoptosis, we investigated the effect on activation of caspase-3. Caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins (Porter and Janicke, 1999). Treatment with rapamycin leads to a nearly 50%

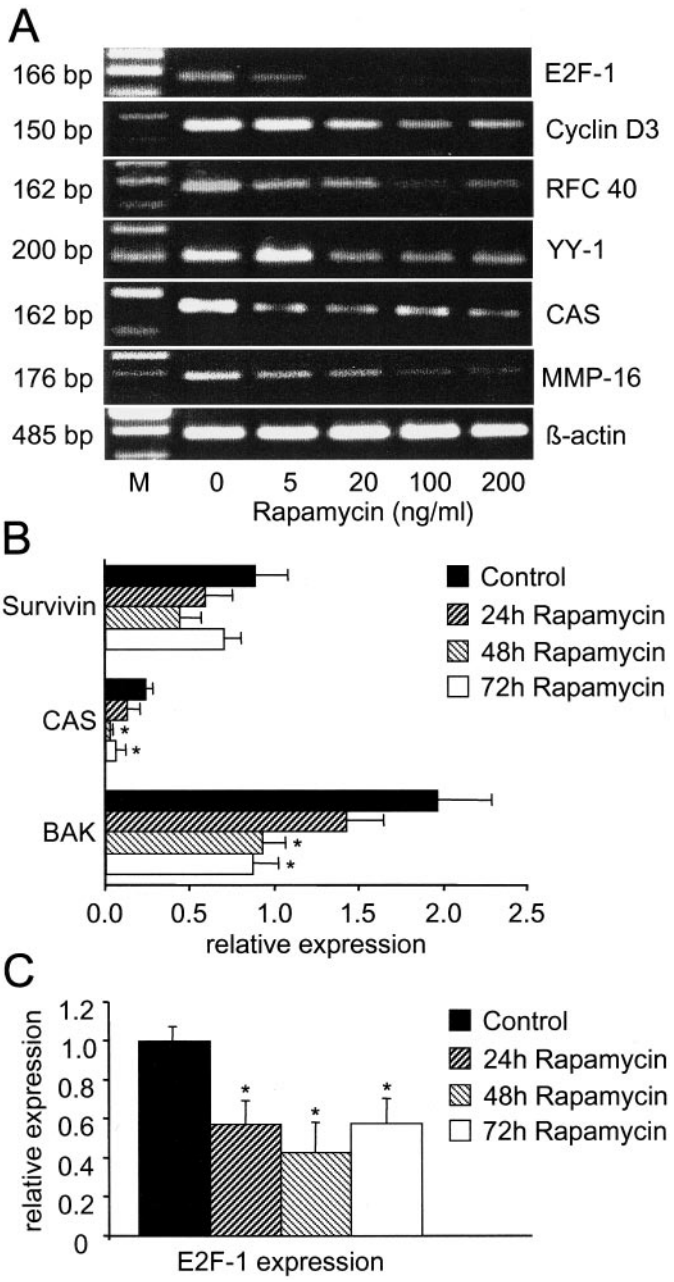

Fig. 4. Validation of gene expression data by PCR. A, verification of dose-dependent down-regulation of mRNA levels by gene-specific PCR after incubation of CASMCs with rapamycin for 48 h. Different concentrations of rapamycin were as indicated. The size of expected PCR fragment is indicated on the right. M, marker band; bp, base pair. B, verification of time-dependent decrease in mRNA expression of apoptosis-associated genes by gene-specific PCR. The results are shown as mean values of three independent experiments ± S.E.M. ⋆, $p < 0.05$. C, verification of time-dependent decrease in E2F-1 mRNA expression by real-time PCR. The results are shown as mean values of five independent experiments ± S.E.M. ⋆, $p < 0.05$.

reduction in caspase-3 activation in proliferating and hydrogen peroxide-treated CASMCs, indicating that rapamycin may at least partially inhibit apoptosis by inhibition of caspase-3 (data not shown).

Rapamycin Reduces CASMC Adhesiveness for Monocytic Cells and EMAP-II Gene Expression. It has recently been demonstrated that an increased neointimal macrophage infiltration correlates significantly with the extent of neointima formation in humans (Farb et al., 2002), indicating that inflammatory processes play an important role during neointima formation. Therefore, we studied the effect of rapamycin on CASMC adhesiveness for monocytic cells. Rapamycin significantly reduced CASMC adhesiveness for monocytic cells by 34% (Fig. 6). The gene expression analysis has revealed a significant down-regulation of EMAP-II, a novel cytokine with an important role during inflammatory cell tissue infiltration (Kao et al., 1994; Knies et al., 1998), by rapamycin. Thus, we hypothesized that EMAP-II may explain the inhibitory effect of rapamycin on adhesiveness of CASMCs. Indeed, the effect of rapamycin on reduced adhesion of monocytic cells to CASMCs could be completely rescued by addition of recombinant EMAP-II. Furthermore, addition of EMAP-II significantly stimulated monocyte adhesion to CASMCs. EMAP-II has been shown to exist as an inactive precursor and is activated by cleavage through caspases during apoptosis. If rapamycin worked via EMAP-II, induction of apoptosis should amplify the adhesive properties of CASMCs; treatment of CASMCs with a general or specific caspase inhibitor should inhibit the adhesive properties of CASMCs as rapamycin did. To further support the hypothesis of decreased EMAP-II release by reduced mRNA

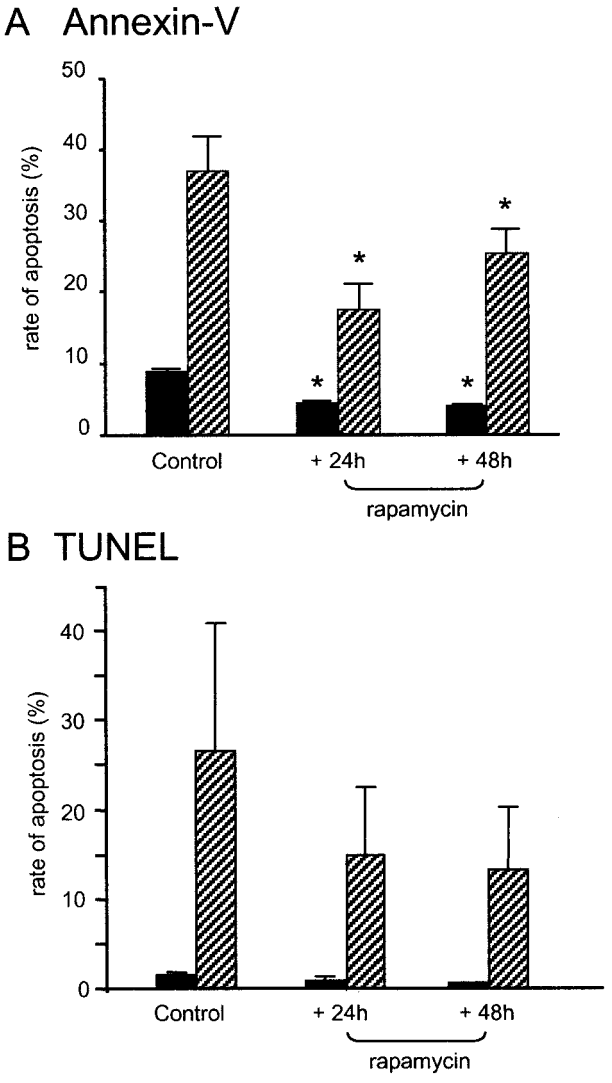

Fig. 5. Effect of rapamycin on survival of CASMCs. Flow cytometry analysis of spontaneous (■) or H_2O_2-induced (▨) (1000 μM for 1 h) apoptosis. Cells were cultured in the presence or absence of rapamycin. A, cells were double stained by annexin-V and propidium iodide at 6 h after induction of apoptosis. The results are shown as mean values of five independent experiments ± S.E.M. ⋆, $p < 0.05$. B, TUNEL staining at 12 h after induction of apoptosis. The results are shown as mean values of three independent experiments ± S.E.M.

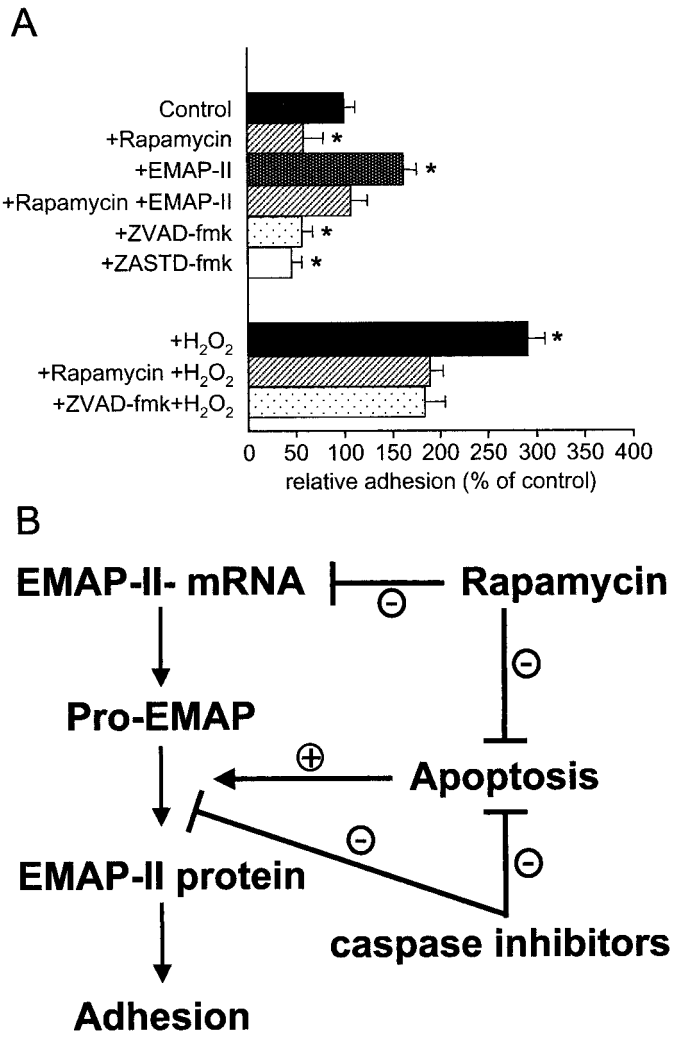

Fig. 6. A, effect of rapamycin on adhesion of monocytic cells. CASMCs were cultured for 24 h in the presence or absence of 100 ng/ml rapamycin or the caspase inhibitor zVAD-fmk (50 μM). Afterward, CASMCs were incubated for 2 h in the presence or absence of 40 μg/ml EMAP-II protein or the specific caspase inhibitor zASTD-fmk (50 μM) before the addition of Mono Mac 6 cells for 30 min. As a positive control, Mono Mac 6 cells were stimulated with EMAP-II protein (40 μg/ml) 2 h before coincubation. In addition, adhesiveness of CASMCs was assessed 6 h after induction of apoptosis with 100 μM H_2O_2 in the presence or absence of rapamycin (100 ng/ml) or the caspase inhibitor zVAD-fmk (50 μM). Adhesion of Mono Mac 6 cells was quantitated by cell counting. The results are a mean value of three independent experiments ± S.E.M. ⋆, $p < 0.05$. B, flow chart clarifying the regulation of active EMAP-II and subsequent effect on adhesiveness of CASMCs by rapamycin and caspase inhibitors.

expression and reduced apoptosis mediating the anti-inflammatory effects of rapamycin, we performed the following experiments: 1) adhesiveness of CASMCs for monocytic cells was assessed in the presence of the general caspase inhibitor zVAD-fmk and the specific caspase inhibitor zASTD-fmk, which has been shown to inhibit cleavage and release of active EMAP-II (Knies et al., 1998); and 2) CASMCs were preincubated with hydrogen peroxide before assessment of adhesiveness in the presence or absence of the caspase inhibitor zVAD-fmk or rapamycin. Indeed, induction of apoptosis by hydrogen peroxide significantly induced the adhesiveness of CASMCs for monocytic cells. Likewise, pretreatment with rapamycin or zVAD-fmk prevented significant alteration in adhesiveness of CASMCs (Fig. 6A). Thus, the observed down-regulation of EMAP-II by rapamycin seems to be functionally involved in the inhibitory effect of rapamycin on the adhesiveness of CASMCs for monocytic cells.

Discussion

Rapamycin reduces the risk of in-stent restenosis by inhibiting neointimal proliferation in humans (Poston et al., 1999; Sousa et al., 2001). However, the underlying mechanisms of how rapamycin governs neointima formation are not yet understood in detail. Here, we systematically investigated the effect of rapamycin on the properties of CASMCs. We show that: 1) mTOR was nuclear translocated and phosphorylated in CASMCs and T cells from human neointima from coronary in-stent restenosis, and that rapamycin 2) regulated the expression of 227 of 2231 genes analyzed, including an pRb/E2F-1–associated gene expression pattern, 3) decreased the rate of basal and H_2O_2-induced apoptosis, and 4) reduced CASMC adhesiveness for monocytic cells.

Expression of mTOR Protein in Neointimal CASMCs and T Lymphocytes. mTOR is a central regulator of cell growth and proliferation, but its role in CASMC growth and neointima formation has not been examined yet. In this study, we show that mTOR protein is expressed in CASMCs in the media of normal as well as neointimal arteries and in T lymphocytes infiltrating the neointimal tissue. We identified an increased nuclear translocation and phosphorylation of mTOR in CASMCs and T lymphocytes of human neointima. The mechanisms that control mTOR activity are still unclear. Activation of the phosphoinositide 3-kinase [PI(3)K]/Akt pathway induces an increase in phosphorylation of mTOR on Ser 2448 (Nave et al., 1999; Reynolds et al., 2002), which parallels the increase in mTOR activity after activation of the PI(3)K/Akt pathway. This suggests that phosphorylation of mTOR on Ser 2448 is of functional relevance and can serve as a marker of mTOR kinase activity. In contrast, activation of the PI(3)K/Akt pathway had no effect on the expression of the mTOR protein (Nave et al., 1999). The PI(3)K/Akt pathway is a key signaling route for proliferative responses to mitogens such as platelet-derived growth factor (Walker et al., 1998), and activation of this pathway is important for CASMC replication after arterial injury (Shigematsu et al., 2000).

In addition, it has recently been shown that nuclear mTOR displays a strongly enhanced kinase activity compared with cytoplasmic or membrane-bound mTOR (Zhang et al., 2002). Likewise, nuclear shuttling of mTOR is essential for its kinase activity, and an increase in nuclear translocation correlates with an increase in mTOR kinase activity (Kim and Chen, 2000). Our findings support the notion that activation of the mTOR kinase plays an important role in CASMC growth during neointima formation in humans and that the effect of rapamycin on neointima formation is caused by its inhibitory effect on mTOR.

Transcriptional Effect of Rapamycin on Cell Cycle Control in CASMCs. We identified 227 of 2231 genes examined (9.8%) that were altered in CASMCs by rapamycin. Almost all of these genes were down-regulated. Because we neither found a higher rate of cell death in rapamycin-treated CASMCs (Fig. 5) nor saw differential expression of housekeeping genes, we conclude that rapamycin-induced down-regulation of gene transcription is not caused by cellular toxicity but represents a unique drug-specific effect leading to silencing of proliferating CASMCs.

However, profound changes could be observed in the expression of genes associated with cell cycle transition. This could explain decreased cell division in rapamycin-treated CASMCs. Rapamycin inhibits hyperphosphorylation of pRb, which is required for the release of sequestered E2F. pRb and E2F are critical regulators of the cell cycle (Harbour and Dean, 2000a,b). Through its interaction with E2F-1, pRb inhibits transcription of genes associated with proliferation and apoptosis. Accordingly, E2F-1 expression was significantly suppressed by rapamycin. Simultaneously, we found down-regulation of 41 pRb/E2F-1-associated genes such as YY1 and cyclin D3. In human CASMCs, YY1 favors progression into S-phase, which can be blocked by activated pRb (Petkova et al., 2001). Our data regarding the effect of rapamycin on E2F-1 gene expression contradict data from Brennan et al. (1999), who demonstrated that rapamycin decreases E2F activity without affecting E2F expression. However, this group performed their experiments in T lymphocytes and investigated the early effects of rapamycin on E2F protein expression. We found that the impact of rapamycin on mRNA expression of E2F-1 was the strongest after 48 h of rapamycin treatment, supporting the concept that protein down-regulation may take place later than inactivation of E2F-1.

E2F-1 and cyclin-D3 are up-regulated in CASMCs from human neointima (Zohlnhöfer et al., 2001b). Compatibly, rapamycin reduced the expression of cyclin-D3 and CDK4. Cyclin-D3 binds to activated pRb and thereby targets CDK4 to pRb. CDK4 then induces inactivation of pRb, leading to cell cycle progression (Kato et al., 1993). In conclusion, our data provide evidence for a role of cell cycle-regulating genes in the inhibition of CASMC proliferation after rapamycin treatment.

Effect of Rapamycin on Apoptosis of CASMCs. Apoptosis of CASMCs has an impact in the vascular response to injury and is involved in the pathogenesis of restenosis (Isner et al., 1995). It precedes proliferation of CASMCs, implying that signals from apoptotic cells may contribute to neointima formation (Malik et al., 1998). Becausereactive oxygen species-induced apoptosis plays a role in neointima formation (Li et al., 1999), we investigated the effect of rapamycin on hydrogen peroxide-induced apoptosis in CASMCs.

Rapamycin led to a reduction in basal and H_2O_2-induced apoptotic cell death in CASMCs compared with nontreated

CASMCs. This finding is reflected in a reduced activation of caspase-3 in rapamycin-treated CASMCs.

Our data are discrepant with those of Roque et al. (2001), who found a slight increase in apoptosis of murine CASMCs after treatment with rapamycin at a concentration of 10 ng/ml. In our experiments, we treated CASMCs with rapamycin at a concentration of 100 ng/ml. Moreover, we could reproduce an increase in the apoptosis rate at a concentration of 10 ng/ml (data not shown). This dose-dependent effect of rapamycin complies with the clinical experience. There, high tissue levels of rapamycin (approximately 100 ng) achieved after implantation of rapamycin-coated stents (Suzuki et al., 2001; Moses et al., 2003) led to a significant reduction of neointima formation, whereas oral treatment resulting in low systemic levels (10–15 ng/ml) did not show any clinical benefit (Brara et al., 2003).

Role of EMAP-II in Mediating Adhesiveness of CASMCs. It has recently been demonstrated that medial injury incurred during angioplasty and stent implantation induces an increased arterial inflammation accompanied by an increased inflammatory cell density in the human neointima (Farb et al., 2002). The increased arterial inflammation is significantly associated with an increase in neointimal growth and restenosis, suggesting that the arterial inflammation plays an important role in the pathogenesis of restenosis.

The reduced apoptosis of CASMCs mediated by rapamycin may contribute to the anti-inflammatory effect of rapamycin during neointima formation. It has been shown recently that inhibition of apoptosis can prevent inflammation and tissue injury (Daemen et al., 1999). EMAP-II evolving from cleavage of the 43kDa pro-EMAP protein was identified as the linking molecule between apoptosis and inflammation (Daemen et al., 1999). In our study, EMAP-II gene expression was reduced by rapamycin. Treatment with rapamycin resulted in reduced adhesiveness of CASMC. The observation that addition of recombinant, endotoxin-free EMAP-II can restore adhesion of monocytes to rapamycin-treated CASMCs strongly suggests that EMAP-II plays a proinflammatory role during neointima formation. The expression of the active EMAP-II protein can be regulated at several points (Fig. 6B). We hypothesized that rapamycin reduces mRNA expression of EMAP-II as well as cleavage of the precursor protein by inhibition of apoptosis, leading to a diminished adhesiveness of CASMCs for monocytic cells. This assumption is supported by the fact that a specific caspase inhibitor for caspase-7 and a general caspase inhibitor significantly reduced adhesiveness of CASMC to monocytes, similar to the observations made with rapamycin. In fact, the specific caspase inhibitor employed has been shown to reduce levels of released EMAP-II (Knies et al., 1998), suggesting EMAP-II as a link between apoptosis and increased adhesiveness of CASMC.

Therefore, our data suggest an additional antiadhesive and thereby anti-inflammatory effect of rapamycin: by inhibiting the expression of the monocyte chemoattractant EMAP-II, rapamycin leads to a decrease in CASMC adhesiveness and thereby may block the early recruitment of inflammatory cells to the injured arteries. This effect may further reduce the vascular proliferative response in humans.

Limitations of the Study. Becausewe focused on the effect of rapamycin on gene expression in CASMCs, we have to consider that rapamycin has profound effects on protein translation and on post-transcriptional regulation of cell cycle regulators (Poon et al., 2002). Rapamycin-induced inhibition of CASMC proliferation is associated with elevation of $p27^{kip1}$ (Poon et al., 2002). However, the effect of rapamycin on neointima formation was not abolished in $p27^{kip1}$-deficient mice (Poon et al., 2002), indicating that there are additional mechanisms responsible for the antiproliferative effect. In our study, we did not see any effect of rapamycin on $p27^{kip1}$ mRNA expression. Becauseeffects on protein translation could regulate expression of $p27^{kip1}$ protein, our finding is not in contrast to those of previous studies.

Our study did not address the inhibitory effect of rapamycin on infiltrating T cells in neointima. We showed that mTOR was expressed in CASMCs and T cells of human neointima, indicating that neointimal CASMCs as well as T lymphocytes may be a target of rapamycin. In a previous study, we provided data suggesting that T lymphocytes and interferon-γ are involved in neointima formation (Zohlnhöfer et al., 2001b). Therefore, inactivation of T lymphocytes by rapamycin may cooperate with antiproliferative effects of rapamycin in reducing neointima formation in humans. In this study, we demonstrated that the mTOR signal transduction pathway plays an important regulatory role in CASMC growth during neointima formation and that rapamycin does not simply inhibit the replication of CASMCs but has pleiotropic effects on basal cellular functions contributing to neointima formation. Therefore, the inhibitory effect of rapamycin on neointima formation seems to encompass proliferation, apoptosis, ECM production of CASMCs, and adhesiveness for monocytic cells.

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