mTOR Inhibitors and Calcineurin Inhibitors Do Not Affect Adhesion Molecule Expression of Human Macro- and Microvascular Endothelial Cells

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Introduction

Endothelial cell (EC) dysfunction/activation is a prominent feature in several diseases ranging from atherosclerosis to transplant rejection. These processes lead to rapid and transient upregulation of proinflammatory molecules, such as adhesion molecules. The expression of E-selectin, vascular and intracellular adhesion molecules (VCAM-1, ICAM-1) in capillaries, and the levels of their soluble forms, correlate with the severity of cellular and humoral rejection [1, 2], and may eventually influence patient and graft survival [3]. Endothelial activation and whereas adhesion of PBMC increased significantly, as described by other papers. In summary, neither calcineurin inhibitors nor mTOR inhibitors activate human micro- and macrovascular EC. Therefore, the investigated drugs are unlikely to contribute to EC activation during transplant-associated vasculopathy.
allograft survival have been greatly improved by multiple immunosuppressive drugs [4].

The impact of immunosuppression on individual endothelial properties has been investigated in several in vitro models [5–7]. However, most of these studies used human umbilical veins, immortalized or animal-derived EC often without considering the functional heterogeneity of EC derived from different origins [8, 9]. The objective of our study was to elucidate the direct impact of mTOR inhibitors (mammalian target of rapamycin) and calcineurin inhibitors (CNI) on EC derived from micro- and macrovascular origins. mTOR inhibitors are important immunosuppressive drugs that exert synergistic immunomodulatory effects when administered together with CNI as they lead to a sequential blockade of IL-2-mediated pathways: CNI lead to a decreased production of IL-2 and mTOR inhibitors block IL-2-activated mTOR signal transduction. Furthermore, a combined regimen of CNI and mTOR inhibitor treatment with dose tapering of the CNI seems to be a clinically efficient strategy, although there is still a lack of data focusing on their effects at specific sites of action. Therefore, we focused on these 2 drug classes. A particular ex vivo–in vitro model using patient-derived EC cultures was applied to closely mimic the in vivo situation of atherosclerotic vessels during the early postoperative period after transplantation with incipient immunosuppression.

Methods

Cell Culture

Patient-derived saphenous vein EC (HSVEC) and coronary artery EC (HCAEC) were isolated from patients undergoing coronary artery bypass surgery and orthotopic heart transplantation. Adult dermal EC, cardiac microvascular EC (HMVEC) and HCAEC were purchased from Cambrex (Walkersville, Md., USA) and Promocell (Heidelberg, Germany). All experiments were approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki. EC cultures were cultivated in endothelial-specific medium (EGM-kit; Cat. No. C-22010, Promocell) supplemented with 30% pooled human serum (culture medium with serum; CMS) according to Lehle et al. [10]. These culture conditions are essential for optimal growth of patient-derived EC in the indicated experimental time frame. The purity of the preparations was 99%, as monitored by staining with an anti-human CD31-PE antibody (Ancell, Bayport, Minn., USA), and flow-cytometric analysis (FACSCalibur, BD Biosciences, San Jose, Calif., USA). EC from passages 3–5 were seeded with a density of 4,000 cells/cm² in 96-well plates (Costar, Pleasanton, Calif., USA) using 150 μl of CMS per well. After 3 days, the medium was renewed and immunosuppressive drugs were added for 48 h under standard culture conditions. In order to detect tumor necrosis factor (TNF)-induced expression of E-selectin, VCAM-1 and ICAM-1, EC were stimulated with 10 ng/ml TNF (Calbiochem, Bad Soden, Germany) for the last 4 h (E-selectin) and 20 h (ICAM-1, VCAM-1) of drug treatment, respectively. In order to exclude any influence of the proliferative activity of untreated control cells, we seeded EC with a density of 30,000 cells/cm² in 96-well plate for 2 days, and incubated with each drug for 48 h as described above. The cells in the resulting confluent monolayer were stable over the next 4 days. Trypan blue exclusion yielded a viability of about 85–95%.

Immunosuppressive Drugs

Cyclosporin A (CsA) and everolimus (Eve) (Novartis Pharma, Nürnberg, Germany), tacrolimus (Tac; Fujisawa Healthcare, Deerfield, Ill., USA), and sirolimus (Sir; Wyeth-Ayerst, Madison, N.J., USA) were dissolved in 100% ethanol (10 mM stock solution) and diluted with ethanol and CMS (working concentration of the drug 1 μM; ethanol always being 0.1%, 150 μl per well). Control cells were incubated with CMS plus 0.1% ethanol.

Cell Growth Characteristics

To determine cell numbers and dimensions, EC were treated as described above, detached by collagenase type 2 (Roche Diagnostics, Basel, Switzerland) treatment and cell suspensions were counted using an automated cell analyzer system allowing both the discrimination of viable cells from dead cells/debris and the determination of average cell volumes (CASY1; Schärfe-System, Reutlingen, Germany). Cell counts were recorded after 48 h of drug exposure and documented in relation to respective untreated controls (100%) at the corresponding time points.

Cell Viability, Mitochondrial Activity and Apoptosis

The metabolic status of EC in culture was determined using the tetrazolium-based mitochondrial dehydrogenase assay (MTS; Promega, Madison, Wisc., USA) according to manufacturer’s instructions. EC were incubated with or without immunosuppressive drugs for 47 h in CMS, which was then replaced by medium (without additives) supplemented with 16% MTS solution and incubated for 1 h under standard culture conditions. Absorbance was measured at 490 nm with a 96-well-plate reader (MWG-Biotech, Ebersberg, Germany) and cellular absorbance was calculated by dividing absorbance per well by cell counts per well.

Apoptosis was determined in cultures (drug treated and untreated) via the activation of caspases-3/7 (Promega) following manufacturer’s instructions. After exhausting the supernatants, EC were lysed by addition of a lysis buffer (including caspase-3/7 substrate) and frozen overnight at –20 °C. Caspase activity was expressed as fluorescence per counted cell. Incubation with 10 μM staurosporine (Sigma, St. Louis, Mo., USA) for 4 h was used as a positive control.

Simultaneously, experimental series were assayed microscopically for cell viability using the trypan blue exclusion technique.

Proliferative Activity

Proliferative activity was assessed using 2 techniques: (1) [Methyl-3H]-thymidine incorporation: [Methyl-3H]-thymidine 0.1 μCi (Amersham Pharmacia, Buckinghamshire, UK) was added per well during the last 24 h of the 48-hour drug incubation period. Cells were dissociated with 2.5% trypsin/1% EDTA (BioWhittaker, Waldersville, Md., USA) and harvested on glass-
and resuspended in PBS (2×). Cells were centrifuged at 400 g for 5 min and resuspended in PBS (2×) containing 2% BSA and fixed in 70% methanol (Merck, Darmstadt, Germany) at 4 °C overnight (1× 10^6 cells/ml). For propidium-iodide staining, cell suspensions were again washed, pre-incubated with 10 μg/ml RNase (Sigma) (37 °C, 20 min), and propidium iodide was added (50 μg/ml, 15–30 min). DNA histograms were recorded using a FACS Calibur flow cytometer. High-quality cell cycle analysis was performed with the WinCycle software package (Phoenix Flow Systems, San Diego, Calif., USA).

Expression of Cellular Adhesion Molecules
Drug-treated and untreated EC monolayers in 96-well plates were washed with pre-warmed PBS, fixed with methanol/acetone (1:1, –20 °C, 10 min), and stored at –20 °C to determine cellular amounts of VCAM-1, ICAM-1 and E-selectin using a cellular ELISA [10]. In a separate experimental setup, the cell count was analyzed under identical culture conditions using CASY1. The expression of cellular adhesion molecules (CAM) was defined as the ratio of absorbance per culture area (0.3 cm²) and the cell count of the respective sample. The activation factor (AF) defines the proportions of TNF-stimulated expression and basal expression.

For histological analysis, EC were grown on chamber slides (Nunc, Roskilde, Denmark) up to confluence, treated with TNF or culture medium for 4 h, washed with PBS, fixed in acetone/methanol (1:1) for 10 min at –20 °C and labeled using an FITC-conjugated monoclonal anti-human CD62E (E-selectin) antibody (Dianova, Hamburg, Germany).

Adhesion of Peripheral Blood Mononuclear Cells
Peripheral blood mononuclear cells (PBMC) were separated from EDTA peripheral blood of healthy volunteers by Ficoll-Paque density gradient centrifugation (density: 1.077 g/ml, Amersham Biosciences, Upsala, Sweden) and washed in PBS. PBMC (1 × 10^6 cells/ml) were stained with Calcein-AM (5 μM, 15 min, 37 °C; Molecular Probes, Eugene, Oreg., USA), washed twice with PBS, centrifuged at 400 g (10 min, room temperature), and resuspended in PBS (2 × 10^5 PBMC/ml). The ratio of the number of stained PBMC and respective fluorescence was calculated to quantify the number of adherent PBMC (standard curve). The adhesion assay was performed as previously described by De Clerck et al. [11]. EC were cultivated in 96-well microtiter plates and incubated with immunosuppressive drugs as described above. For the last 4 h of incubation with drugs, EC were treated with 10 ng/ml TNF or PBS as a control. Afterwards, EC were washed with pre-warmed CMS and incubated with 1.2 × 10^6 labeled PBMC per well at 37 °C for 30 min. Nonadherent PBMC in the supernatant were removed by washing 3 times. The fluorescence of the adherent PBMC was measured (excitation 485 nm, emission 535 nm).

In a separate set of experiments, the number of EC were measured with CASY1 and the ratio of adherent PBMC (via standard curve) to EC was determined.

Statistics
Data are given as mean (quadruplicate samples for each culture) ± SD. Wilcoxon signed rank test was used to verify differences between drug-treated and untreated control cells. Most parameters were documented in relation to respective untreated controls. The effect of different drugs on EC was analyzed using the Mann-Whitney rank sum test. Statistical significance was considered to be p < 0.05. p < 0.01 was considered to be highly significant; results are marked with 1 or 2 asterisks within the graphs, respectively.

Results
Impact of Immunosuppressive Drugs on EC Viability and Proliferation
High concentrations of Tac (1 μM) did not affect viability (trypan blue staining, MTS test) and absolute cell count in the monolayers of micro- and macrovascular EC. However, the incorporation of [methyl-3H]-thymidine was reduced for HCAEC. Reduction of the drug concentration in the culture medium (0.1–100 nM) did not influence the amount of EC in the monolayer compared to untreated control cells (data not shown). At the same concentration CsA did not influence cell growth of HSVEC and HMVEC. However, HCAEC showed a higher sensitivity to this CNI. Compared to untreated control cells, incubation with 1 μM CsA reduced cell counts significantly by about 20–30% (fig. 1). The effect was verified by measuring the incorporation of [methyl-3H]-thymidine (not significant, fig. 1). The mitochondrial activity per remaining cell (MTS reaction) was not influenced by drug treatment (data not shown). The effect of CsA on the cell growth of HCAEC was dose dependent. Thus, incubation of HCAEC with low and therapeutic concentrations of CsA (0.1–100 nM) did not affect the cell count in the monolayer (data not shown). In addition, in a preliminary experiment we showed that the time to reach the maximum drug effect was more than 36 h (t½ being 29–30 h, t½ reflecting the time at which half maximal inhibition of cell growth was attained). Therefore, we fixed an experimental time of 48 h, as mentioned above. High concentrations (1 μM) of Sir and Eve delayed cell growth. After 48 h of treatment the cell density was about 40% lower than untreated controls (HSVEC: p < 0.01; HCAEC and HMVEC: p < 0.05; fig. 1a). Furthermore, drug exposure did not systematically modify EC volume (e.g. HSVEC: untreated controls: 4,360 ± 545 fl; CsA: 4,520 ± 765 fl; Tac: 4,250 ± 695 fl; mTOR and Calcineurin Inhibitors and Endothelial CAM Expression

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Sir: 3,895 ± 570 fl; Eve: 3,980 ± 580 fl). Reduced cell growth in cultures treated with mTOR inhibitors was accompanied by a significantly reduced overall [methyl-3H]-thymidine uptake as opposed to the controls, which indicates mTOR inhibitor-induced cytostasis (HSVEC: p < 0.05 for Eve; HCAEC: p < 0.01 for Eve and Sir; HMVEC: p < 0.05 for Eve and Sir; fig. 1b). Using therapeutic concentrations (CsA: 0.1 μM; Tac, Sir and Eve: 0.01 μM), the impact on EC viability and proliferation described above was confirmed.

**Impact of mTOR Inhibitors on Cell Cycle and Potential Apoptosis Induction**

The inhibition of cell growth by mTOR inhibitors was obviously not due to an induction of programmed cell death, as verified via a caspase-3/7 activity assay (fig. 2a), but clearly resulted from the critical reduction in proliferative activity, of about a 50% decrease in S-phase fraction, determined by flow-cytometric DNA histogram analysis (fig. 2b).

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**Impact of Immunosuppressive Drugs on the Expression of CAM**

To elucidate the effect of immunosuppressive drugs on the expression of CAM by EC grown to confluence, monolayers of EC were exposed to high concentrations (1 μM) of CsA, Tac, Sir, and Eve for 48 h. With respect to the anti-inflammatory activity of mTOR inhibitors (see above), the expression of CAM per cell was calculated. As shown in figure 3a–c, none of the CNI or mTOR inhibitors affected the basal cellular expression of CAM of any of the studied cell types. Stimulation with TNF resulted in a significant increase in the expression of each CAM (p < 0.01). The increased expression of E-selectin for example, was shown in a representative fluorescence staining in figure 4a. The activation of the cells with TNF was verified in figures 3d–f as the ratio of the absorbance after TNF stimulation and the basal absorbance. This activation factor (AF) was independent of the pretreatment with CNI and mTOR-inhibitors. However, under control conditions the AF of ICAM-1 and E-selectin were significantly higher for HMVEC than for EC of other origins (fig. 3d–f).

**Impact of Immunosuppressive Drugs on the Adhesion of Leukocytes**

The upregulation of CAM could be verified by the adhesion of PBMC to the surface of drug-treated EC. Stimulation of EC with TNF resulted in a significant increase in the adhesion of freshly prepared PBMC as shown for HSVEC in figure 4b. While CsA and Tac did not affect the adhesion of PBMC to EC, incubation of EC with high concentrations of both mTOR inhibitors (1 μM) resulted in a significant increase in TNF-induced PBMC adhesion (fig. 4b). The other cell types provided analogous data (data not shown).
Impact of Immunosuppressive Drugs on CAM Expression of EC Seeded in a Hyperconfluent Monolayer

To exclude any influence of the proliferative activity of untreated control cells, another set of experiments was repeated with a hyperconfluent monolayer known to auto-inhibit cell proliferation and as a result leave cell counts virtually unchanged. As shown in figure 5a, long-term drug treatment (48 h) of nonproliferating HSVEC did not start an induction of E-selectin (control: 66,000 ± 27,825 cells/cm²; CsA: 77,000 ± 29,300 cells/cm²; Tac: 70,400 ± 28,800 cells/cm²; Sir: 60,450 ± 24,300 cells/cm²; Eve: 57,200 ± 23,900 cells/cm²; not significant). TNF was used as a positive control for the stimulation of untreated control cells. In addition, preincubation of HSVEC with drugs (44 h) did not affect the TNF-induced expression of adhesion molecules. The AF was independent of drug disposure (fig. 5b). The other cell types provided analogous data. According to the expression of E-selectin, drug treatment showed no influence on the basal-induced and TNF-induced expressions of ICAM-1 and VCAM-1 (data not shown). However, drug treatment of EC affected the TNF-induced adhesion of PBMC in a different way. While CNI did not affect the PBMC adhesion on HSVEC (CsA: 90 ± 60%, Tac: 133 ± 68% of untreated control value), preincubation of EC with mTOR inhibitors (1 µM) resulted in an increase in the amount of adherent PBMC on EC (Sir: 134 ± 70%, Eve: 171 ± 77% of untreated control value). Due to the high interindividual variability of each cell culture, the differences were not significant. This could be detected for the other cell types (data not shown).

Discussion

This study directly compares different immunosuppressive drugs under identical culture conditions regarding their potential to modulate micro- and macrovascular endothelial cell properties. The essential new findings are that: (1) Sir and Eve inhibited proliferation of both macro- and microvascular EC, whereas CsA only reduced cell growth of coronary artery EC, and Tac showed generally no antiproliferative activity but exclusively reduced [methyl-3H]-thymidine incorporation of HCAEC; (2) none of the immunosuppressive drugs induced ex-
expression of the investigated adhesion molecules; (3) mTOR inhibitors increased the TNF-induced adhesion of PBMC.

In our experiments, high concentrations of CsA and Tac exclusively exerted antiproliferative effects on HCAEC in contrast to EC from other vessels. The effect disappeared at lower and therapeutic concentrations (0.1–10 nM) of both drugs. Dose- and time-dependent responses to CNI treatment have already been described for diverse nonendothelial and endothelial cell types [12–14]. While the cell growth of human late-outgrowth endothelial progenitor cells and mature aortic EC was not affect-
ed by high concentrations of both CNI [13, 14], CsA reduced viability of EC from the blood-brain barrier [15] and medullary thick ascending limb cells [16]. In contrast, Tac did not affect the cell proliferation of these cell types [12, 13, 15, 16]. On cellular levels, previous studies reported both enhanced proliferation at low concentrations [6] and decreased cell growth at high concentrations [12–14, 17, 18]. The usage of different EC types (venous, aortic, corneal, microvascular, endothelial progenitor cells or immortalized cell lines) and different incubation times made a comparison of these data difficult. In our present study, all EC types were treated with the same concentration of CsA under identical culture conditions. Therefore, it is conceivable that the different responses to CsA depended on the higher sensitivity of HCAEC for CsA. However, therapeutic concentrations of CsA (100–300 nM) and of Tac (6–24 nM) did not affect the cell density of drug-treated EC monolayers.

For functional analysis, we demonstrated that none of the CNI exhibited an anti-inflammatory effect in our culture system – not even at peak concentrations. CsA and Tac neither affected the release of IL-6 (data not shown) nor the expression of CAM and the adhesion of PBMC. In contrast to other scientific groups, our experi-

**Fig. 4.** Expression of E-selectin and PBMC adhesion of drug-treated and untreated HSVEC. a Immunofluorescent staining of a representative HSVEC culture using a FITC-conjugated anti-CD62E antibody. Basal expression (left picture) and TNF-induced expression after 4 h (right picture) of control cells. b HSVEC (n = 5) were pretreated with CsA, Tac, Sir, Eve (1 μM) and the medium (control) for 44 h and then incubated with PBS (left picture) and TNF (right picture) for 4 h. Freshly prepared PBMC were incubated with drug-treated and untreated EC, and the amount of adherent PBMC per cell was determined. *p < 0.05, comparing drug-treated versus untreated EC.
mental setup should mimic steady-state conditions with an acute episode of TNF stimulation, as possibly seen under in vivo conditions after organ transplantation. Other scientific groups described different effects of CsA. Charreau et al. [5] and Markovic et al. [19] showed, for example, that CsA downregulated the expression of E-selectin and VCAM-1 under concomitant stimulation with TNF, while the expression of ICAM-1 remained unchanged [19, 20]. Furthermore, Rafiee et al. [21] demonstrated that CsA enhanced leukocyte binding by human intestinal microvascular EC (HIMEC) through inhibition of p38 MAPK and iNOS. They used HIMEC to provide a mechanistic explanation regarding a proinflammatory effect of CsA on the organ-specific microvascular endothelium, and to demonstrate the failure of this compound in the long-term treatment of chronic inflammatory bowel disease. We speculate that the different proinflammatory responses could be reduced to the organ-specific heterogeneity of EC [8] or different culture conditions. Karlsson and Nassberger [22] confirmed our finding that Tac does not affect EC activation in response to inflammatory stimuli like TNF. Moreover, up to supratherapeutic Tac levels, there seem to be no deleterious effects on capillaries in vitro [7].

In contrast, mTOR inhibitors inhibit such proliferation of both hematopoietic and nonhematopoietic cells, showing their action as potent immunosuppressive drugs [23, 24]. Inhibition of smooth muscle cell proliferation is especially relevant for protection against cardiac allograft vasculopathy (CAV) [25]. Moreover, one has to keep in mind the participation of endothelial activation/dysfunction in the process of CAV [26]. To demonstrate the impact of mTOR inhibitors on the activation status of the endothelial layer after transplantation or stent implantation, we documented a significant decrease in EC growth within the therapeutic range of immunosuppressive efficacy of Sir and Eve. Previous studies agreed with our data and speculated that the mechanism of cell death appeared to be apoptosis induced by caspase-3 activation [27–29]. However, our data, as well as those of Miriuca et al. [13], clearly showed no caspase-3 activation under mTOR inhibition. Instead, we observed cell-cycle arrest without a cytotoxic effect. We speculate that the therapeutic approach of mTOR inhibitors to reduce intimal hyperplasia by inhibition of smooth muscle cell proliferation is paralleled by a reduced ability to maintain vascular reendothelialization. Thus, inhibition of EC proliferation by mTOR inhibitors indicates that Sir and Eve delay reendothelialization after balloon dilatation and combined introduction of drug-eluting stents, as seen in humans, 3 weeks after stent deployment [13, 30, 31].

The central event in the development of CAV is the inflammatory response to immune- or non-immune-mediated endothelial damage, which is also characterized by the upregulation of adhesion molecules and the subsequent binding of leukocytes [25]. Despite the absence of acute inflammatory processes in heart transplant patients, the concentration of circulating adhesion mole-
cules has been reported to be enhanced [32]. With our culture system, we are able to mimic such steady-state conditions to evaluate the specific effects of immunosuppressive drugs on EC stimulation and to study the effect of acute inflammatory events on the activation status of EC. We could show that long-term incubation of micro- and macrovascular EC with both mTOR inhibitors had no effect on the basal release of IL-8 and MCP-1 [27], as well as on the expression of CAM. Due to different experimental protocols in the literature [5–7, 13, 14], using hyperconfluent seeding of EC for 24 h accompanied by drug treatment for an additional 24–48 h, we validated our previous data in a confluent monolayer experiment. The drug effects were independent of the seeding protocol. Furthermore, we recently reported on an inhibition of basal IL-6 synthesis of HCAEC by both mTOR inhibitors [24], which could not be detected for HSVEC and HUVEC. We concluded that Sir and Eve per se do not result in activation of the endothelium. Specific anti-inflammatory and anti-inflammatory cytokine effects of Sir were also documented in recent studies. Due to different stimulation mechanisms compar-

TNF stimulation of our micro- and macrovascular EC enhanced expression of CAM and the adhesion of leukocytes. The increased density of E-selectin and VCAM-1 on microvascular EC was also documented by Stins et al. [36]. We postulate a cell type-specific heterogeneity of our EC [8]. In our study, none of the mTOR inhibitors influenced the TNF-induced expression of CAM. Para-

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In summary, treatment of EC with mTOR inhibitors delayed reendothelialization in a dose-dependent manner [24]. However, our data also show that neither calcineurin inhibitors nor mTOR-inhibitors activated EC per se. This suggests that the chronic inflammatory process after transplantation [32] is not triggered by the specific effects of immunosuppressive drugs on the EC monolayer. Anti-inflammatory effects of mTOR-inhibitors may also be beneficial in future clinical trials by allowing a withdrawal or dose-reduction of CNI in the immunosuppressive regimen after cardiac transplantation.

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