Original Article

Effects of rapamycin and tacrolimus on mature endothelial cells and endothelial progenitor cells

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Abstract

Objective: To investigate the effects of the potent immunosuppressive agents tacrolimus and rapamycin on the number of circulating mature endothelial cells and circulating endothelial progenitor cells in an experimental model.

Methods: It was an experimental study performed from December 2007 to January 2008 in which the effects of the immunosuppressive agents tacrolimus and rapamycin on endothelial progenitr cells and circulating mature endothelial cells were analysed on 24 male wistar albino rats in a controlled environment model. Circulating cell populations were measured by flow-cytometric analysis. Maun-Whitney U test and analysis of vartiance were used for statistical purposes.

Results: Rapamycin increased the number of circulating mature endothelial cells approximately 2-fold compared to tacrolimus. The number of endothelial progenitor cells also was increased in the peripheral blood of rats treated with rapamycin compared to those treated with tacrolimus.

Conclusion: The study showed that treatment with rapamycin is associated with an increase in endothelial progenitor cells and circulating mature endothelial cells. This increase may be associated with endothelial cell damage and repair.

Keywords: Rapamycin, Tacrolimus, Mature endothelial cells, Endothelial progenitor cells. (JPMA 62: 822; 2012)

Introduction

The risk of cardiovascular disease in renal transplant patients is higher than in the general population. Currently, coronary artery disease (CAD) is one of the most important causes of mortality and morbidity in patients with renal disease.[1-3] Endothelial dysfunction plays an important role in the pathogenesis of cardiovascular diseases. Among the potential causative factors involved in uraemic endothelial dysfunction, the role of endothelial progenitor cells (EPCs) has recently been a subject of intensive research. EPCs are bone-marrow derived, monocyte-like circulating mononuclear cells with the ability to adhere to the damaged vessel wall and replace/shed endothelial cells.[4] It is known that EPCs circulate in very low numbers in the peripheral blood and respond to local and systemic stimuli to be recruited at the site of the vascular damage. There is evidence that EPCs may be involved in the process of endothelial maintenance and neovascularisation.[4-7] Functional early EPC is charactarised by three markers: CD133, CD34 and vascular endothelial growth factor receptor-2 (VEGFR-2) which is also called kinase insert domain receptor (KDR) or Flk-1. In the peripheral circulation of adults, more mature EPCs are found that obviously have lost CD133, but are positive for CD34 and VEGFR-2.[8]

Circulating mature endothelial cells (CECs) may appear in the circulation by detaching from activated or damaged vessels and express CD34 and Flk-1, but, unlike the EPCs, they are negative for the haematopoietic marker CD45.[9] CECs also express endothelial markers such as CD146 and CD31. An increase of CECs has been described in several pathological conditions that involve vascular injury or instability. The enumeration of CECs released in peripheral blood usually represents a direct exploration of the endothelium.[10]

Immunosuppressive agents are necessary to prevent graft rejection after solid organ transplantation. However, they are also known to have significant side effects, including endothelial toxicity. Rapamycin has a unique mechanism of action compared to other immunosuppresants, inhibiting mammalian Target Of Rapamycin (mTOR), whereas cyclosporin A (CsA) and tacrolimus (FK506) inhibit calcineurin. Interestingly-although rapamycin and FK506 are related structurally (both are macrolides) and both bind to the intracellular protein FKBP12, they inhibit divergent pathways. Recent studies have shown that mTOR inhibition induces cell death in peripheral blood mono-nuclear cells cultured to favour the outgrowth of endothelial progenitors. The mechanism of cell death appears to be apoptosis and inhibition of survival

signals given by growth factors required by these cells.[11]

Immunosuppressive treatments of patients may directly affect the endothelial function. However, the exact role of the EPC and CEC counts is stil controversial. Few studies published during the last several years have focused on the role of the different immunosuppressive drugs on endothelial biomarkers, and these have all been inconclusive in terms of such an association.[11-21] Therefore, in this study, we aimed at comparing the impact of two theraupetic immunosuppressive agents, rapamycin and FK506, on endothelial functions in an experimental rat model. Endothelial effects were determined by measuring CECs and EPCs.

Material and Methods

The study comprised 24 male wistar albino rats each weighing 300-350 grams. They were housed in cages in a temperature and light-controlled environment and were allowed free access to water. The rats were divided, into 3 groups of 8 each. The animals in Group 1, which served as the control, were treated with vehicle alone (0.09% serum physiologic 0.5cc both intraperitoneal and oral administration). Rats in Group 2 received oral rapamycin (1 mg/kg/day), while intraperitoneal injections of FK506 (1 mg/kg/day) were given to Group 3. After 28 days, each rat was anaesthesised by intraperitoneal pentobarbital (50 mg/kg). The abdomen was opened up through a midline incision; the inferior vena cava was catheterized and a 4 cc. blood sample was obtained from each animal for the flow-cytometric analyses of EPCs and CECs. The study was performed at Marmara Univesity School of Medical between December 2007 to January 2008.

After obtaining blood from each animal, polymorphonuclear cells (PMN) were isolated by density gradient centrifugation. EPCs and CECs were identified from PMN cells by flow-cytometry. Flow-cytometric analyses were performed on a FACS Calibur flow-cytometer. For the flow-cytometric analyses, 100 µl of isolated cells were stained with one of the following antibody panels for each rat: (1) FITC conjugated rabbit anti-rat Flk-1(Santa Cruz biotechnology), PeCy7 conjugated mouse monoclonal CD34 (Santa Cruz biotechnology) and PE-conjugated mouse monoclonal anti-rat CD146 (R&D Systems). (2) PeCy7 conjugated mouse monoclonal CD34 (Santa Cruz biotechnology), seconder PE antibody conjugated goat polyclonal anti-rat CD133 (Santa Cruz biotechnology) and FITC conjugated mouse anti-rat CD45 (Bioscience). To confirm the phenotype, the expression of surface proteins was measured by flow-cytometric analysis. Cells expressing CD34/CD133/CD45 were identified and quantified as progenitor population. Cells staining positive for CD34/Flk-1/CD146 were judged to be CECs. In each analysis, 500000 events (cells) were counted. Absolute numbers of EPCs and CECs (cells/mm3) of each subject were quantified.

Statistical analyses were performed using one-way analysis of variance (ANOVA) and results were expressed as mean±SD. Differences in variables between the two groups were compared using the non-parametric Mann-Whitney U test. Differences between variables were considered statistically significant if the p-value was <0.05.

Results

No significant difference was found between weight and haematologic parameters of the rats in three study groups at the beginning of the study. Haemoglobin levels, however, were lower in FK506-treated group than in the control and rapamycin-treated groups at the end of the study. The difference was statistically significant (Table-1).

The effects of rapamycin and FK506 on EPC and CEC numbers were noted (Figure-1). Mean FK506 and rapamycin plasma levels were 12 ng/ml and 10 ng/ml, respectively (clinical serum concentrations: FK506 5-15 ng/mL; rapamycin 5-10 ng/mL). The flow-cytometric quantification of mature CEC showed significantly ($p<0.05$) elevated amounts of CEC in the rapamycin-treated group compared to the control and FK506-treated groups (171.42±5.67, 151.04±11.44 and

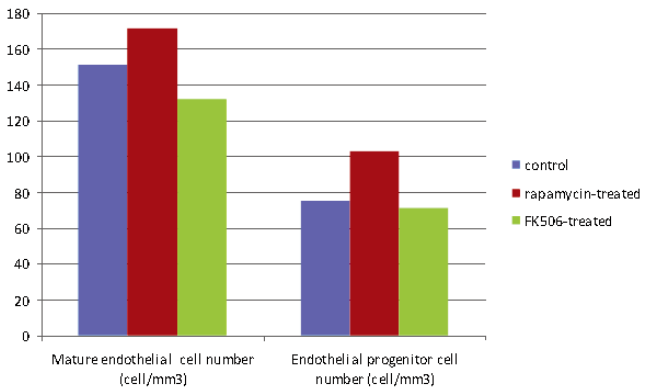

Figure: Number of circulating mature endothelial cells and endothelial progenitor cells.

Table: Comparison of weight profiles and haematologic parameters at the end of the study.

	Control group	Rapamycin-treated group	FK506-treated group	p
Weight (gm)	370±18	380±15	368±12	>0.05
Leucocyte counts (cell/mm3)	6780±2174	6860±1489	7730±2194	>0.05
Haemoglobin counts (mg/dl)	14.1±2.16	15.1±0.64	10.83±1.05	*<0.05

* Hemoglobin counts were lower in FK506-treated group than control and rapamycin-treated groups.

132±8.72 respectively). The flow-cytometric quantification of EPC also showed significantly ($p<0.05$) elevated amounts of EPC in the rapamycin-treated group compared to the control and FK506-treated groups (103±6.85, 75±4.11 and 71.97±9.9 respectively). No difference was found between Group 1 (control) and Group 3 (FK506-treated) in terms of CEC and EPC numbers (p: 0.28 and 0.68, respectively).

Discussion

To our knowledge, this is the first experimental study comparing the effects of different immunosuppressive agents on CECs and EPCs. The main finding of this study was that rapamycin treatment resulted in significantly higher numbers of CECs and EPCs than FK506, which can be a sign of endothelial damage caused by rapamycin.

A study showed that renal transplant recipients (RTRs) without calcineurin inhibitors have significantly lower numbers of CECs than their matched counterparts who received cyclosporine.[12] It also reported a significant relationship between cyclosporine -but not tacrolimus - trough blood levels. In vitro CsA inhibits endothelial cell replication and induces the formation of cytoplasmic vesicles and nucleolar changes. Until recently whether or not FK506 causes as many endothelial side effects as CsA was unknown. Therefore, recent studies have focused on the endothelial effects of FK506. One study compared two different immunosuppressive regimens (CsA/Aza vs. Tac/MMF) by providing simultaneous measurements of CECs, endothelial microparticules (EMP) and sVCAM-1, which provide information about endothelial damage.[13] At the end of the study, two immunosuppressive regimens were found not comparable in terms of endothelial toxicity. Within RTRs, both therapeutic groups exhibited higher numbers of CEC compared to healthy controls. Nine months after transplantation, patients from the CsA/Aza group presented significantly lower levels of CEC than the Tac/MMF group.[13] Another study also demonstrated similar findings.[14] It investigated 95 RTRs: 14 patients were on a calcineurin inhibitor-free immunosuppressive regimen, 48 received CsA, and 33 received FK506. The three groups of RTRs had significantly elevated number of CECs compared with healthy controls. In addition, median CEC numbers were significantly higher in patients receiving FK506 compared with patients on CsA treatment. In our study, FK506 had no significant effect on the number of CECs compared to the control group, indicating that FK506 may not be associated with endothelial damage at this dosage and with the duration of time in our study. Previous studies have shown higher CECs in RTRs receiving FK506.[13,14] The inconsistency between those studies and our own findings most likely reflect small sample sizes and relatively short duration of immunosuppressive therapy which was 28 days only. Low haemoglobin values also were detected in rats exposed to FK506. It was most probably due to FK506-induced nephrotoxicity.

It has been shown that the graft function is an important determinant of EPC number and function in RTRs. A study showed increased number of EPCs in RTRs receiving immunosuppressive treatments when compared with controls.[15] At the time of the study, 68% of the patient cases were on calcineurin inhibitor, and 32% were mostly on mycophenolate mofetil and sirolimus. Similarly, another study found a positive correlation between renal function and EPC level.[16] On the other hand, one study showed that the concentration of circulating EPCs was significantly reduced in RTRs compared with controls.[17] At the time of the study, the RTRs were receiving either CsA (n=43) or FK506 (n=51). Although a study demonstrated reduced EPC function,[17] another one observed improved EPC function in RTRs when compared with controls.[18] In this study, immunosuppressive regimens consisted of calcineurin inhibitors in all patients combined with mycophenolate mofetil and steroids. In the present study, there was no change in the number of circulating EPCs which responded to local and systemic stimuli to be recruited at the site of endothelial damage compared with the controls. These contradictory results might be related to the different methodologies and markers employed in the study of EPC numbers and functions.[15]

As both calcineurin inhibitors and mTOR inhibitors remain the mainstay of immunosuppressive therapy, we also assessed the effects of rapamycin on the same cell types. Rapamycin traditionally is thought of as a proliferation inhibitor because the inhibition of lymphocyte proliferation is recognised as its mechanism of action as a clinical immunosuppressant. However, there are multiple mechanisms by which rapamycin also may be cytotoxic, at least for certain cells. First, there is in vitro evidence of apoptosis induced by caspase activation. The link between mTOR inhibition and caspase activation recently has been suggested by the findings of one study.[22] Another study also showed that exposing endothelial cells to rapamycin induced caspase 3 activation.[23] A second possible mechanism that may contribute to the cell death induced by rapamycin is the suppression of survival signals like ribosomal p70 S6 kinase, BIM and P13K/AKT.[11] Rapamycin also has been reported to increase proapoptotic signals.[11]

Recently different groups have examined the effects of rapamycin on EPCs, late outgrowth endothelial progenitors and mature aortic endothelial cells. A study pointed out that rapamycin induced rapid cell death even at concentrations much lower than those used clinically in peripheral blood mononuclear cells (PBMC) cultured to favour the outgrowth of endothelial progenitors.[11] Cyclosporin A and FK506, however, had no significant effects in clinical concentrations. The effect of rapamycin was found to be specific to EPCs. A lesser effect was observed in late outgrowth endothelial progenitors, mature aortic endothelial cells and macrophages derived from the same PBMCs.[11] Our study is the first in the literature to

examine the effects of rapamycin on EPCs and CECs in vivo. In the present study, rapamycin treatment resulted in significantly higher numbers of EPCs and CECs. Previous studies examined the effects of rapamycin on cultured cells in vitro.[11,19] These studies demostrated a significant reduction in the number of EPCs with no effect on mature endothelial cells. They suggested that mature endothelial cells are protected from cytotoxicity from rapamycin due to limited amounts of intracellular receptor for rapamycine.[11,19] Recently researchers studied the effect of rapamycin on human endothelial cells.[20] In this study, human umbilical vein or aorta endothelial cells were exposed to rapamycin. After a 24-hour period of incubation, rapamycin was found to have caused significant cell loss associated with the increase of both apoptosis and necrosis. The study concluded that prolonged treatment with rapamycin impairs endothelial function. Endothelial damage seems dependent on mTORC2 inhibition. In the present study, in vivo administration of rapamycin resulted in endothelial damage, possibly due to apoptosis and necrosis of endothelial cells as demonstrated by an earlier study.[20] As described previously, CECs may appear in the circulation by detaching from damaged vessels. And that is why we observed significantly higher numbers of CECs. There also were increased numbers of EPCs in our study. We think that EPCs responded to rapamycin-induced endothelial damage in order to be involved in the process of endothelial maintenance and neo-vascularisation.

It is well known that EPC function is as important as EPC number. Recent evidence has demonstrated that rapamycin not only causes cell death, but also inhibits proliferation and differentiation of EPCs in vitro. Researchers have developed a culture system that allowed expansion and endothelial differantiation of human CD133+ precursor cells.[21] In that study rapamycin inhibited the proliferation of CD133+ cells dose dependently; furthermore, the development of adherent endothelial cells from expanded CD133+ cells was also dose dependently inhibited. In the same study, apoptosis induced by rapamycin after 48 hours of treatment was reduced by pre-incubation with tacrolimus.[21] Unfortunately we were unable to evaluate the function of circulating progenitor cells. This is the major limitation of our study.

Conclusion

The study demonstrated that treatment with rapamycin impairs endothelial cell function and results in significantly higher numbers of CECs and EPCs the functional capacities of which are not known exactly. The study also demonstrated that FK506 is not associated with endothelial damage at the dosage and in the time period used in the study.

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