

THE EFFECT OF IMMUNOSUPPRESSION AGENTS ON STEM AND ENDOTHELIAL CELLS' PROLIFERATION IN VITRO

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Abstract

The control of immune-mediated transplant rejection by immunosuppressive agents might affect other human cells' proliferation and function. This study aims to evaluate the effect of three immunosuppressants on the proliferation of human adipose-derived adult stroma (ADAS) and human umbilical vein endothelial cells (HUVEC). These cells were used as a culture model to study the minimum inhibitory dose of commonly used immunosuppressants. Cells were cultured and seeded into 96-well plates at a density of 2,500/well then treated either with one of two calcineurin inhibitors, namely FK506 (Tacrolimus) and Cyclosporin A (CsA), or with the IMP dehydrogenase inhibitor, Mycophenolic acid (MPA). After 5 days, the medium was removed and plates were stored at -70°C until analyzed with the CyQUANT cell proliferation assay. The minimum inhibitory dose on HUVEC proliferation was 1000 ng/ml for CsA, 500 ng/ml for MPA, and 10,000 ng/ml for FK506. The minimum inhibitory dose on ADAS proliferation was 5 ng/ml for MPA, 100000 ng/ml for FK506 and 100000 ng/ml for CsA. Our results suggest that these immunosuppressants inhibit ADAS and HUVEC proliferation in a dose-dependent manner in vitro. The FK506 and CsA minimum inhibitory concentrations on both HUVEC and ADAS were several orders of magnitude greater than those required for T cell inhibition and, therefore, regular dosages are not expected to impair HUVEC or ADAS proliferation. However, the minimum inhibitory concentration of MPA on ADAS was lower than that observed in vitro for lymphocyte function and, therefore, could be of concern.

Experimental article (J Int Dent Med Res 2015; 8: (3), pp. 116-122)

Keywords: Immunosuppression agents, allotransplantation, FK506, cyclosporin A, mycophenolic acid.

Received date: 15 October 2015

Accept date: 13 November 2015

Introduction

The evolution of composite tissue allotransplantation recently has dramatically improved the well being of many patients who had no other reconstructive option. With the help of facial transplantation, it has become possible to achieve an optimal anatomical reconstruction with significant improvement in the functional status.^{1,2} However, the risks of composite tissue

allotransplantation involve mandatory lifelong immunosuppression. Immunosuppressive agents are the front line in limiting rejection following transplantation. The control of immune-mediated transplant rejection conventionally aims at the regulation of clonal expansion of effector T cells and the most commonly used immunosuppressive agents inhibit different steps of T-cell activation.³ Although it is likely that these agents might affect other human cells' proliferation and function, this possibility remains relatively unexplored.

In the field of maxillofacial surgery, immunosuppressive drugs such as Rapamycin, Cyclosporin A (CsA) and Tacrolimus (FK506) has improved efficacy of inhibiting graft rejection and treating many autoimmune diseases like pemphigus, pemphigoid and reluctant cases of oral lichen planus. However, these drugs are not

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very specific and are associated with side-effects and toxicities.²

The dose for an immunosuppressive drug has to be assessed carefully to ensure that the blood concentrations are kept within the target range, and then monitored to allow for proper dose adjustments if required. Therapeutic drug monitoring of immunosuppression agents has been hindered by lack of standardization between proficiency testing programs and inter-laboratory variability. Also the blood/plasma based measurements of the drug concentrations are inaccurate as they ignore the drug concentrations at the site of action and the concentrations of the pharmacologically active moieties.^{4,5}

Mycophenolate Mofetil is an immunosuppressive agent used in combination therapy for the prevention or treatment of acute rejection after solid organ transplantation. It is a broad-spectrum acting drug having antiviral, antifungal, antibacterial, anticancer, and antipsoriasis properties.⁶ Mycophenolate Mofetil is metabolized to its active metabolite, mycophenolic acid (MPA), which acts by inhibiting de novo biosynthesis of purines. MPA is a potent inhibitor of inosine monophosphate dehydrogenase (IMPDH), a key enzyme in the synthesis of Guanosine triphosphate (GTP).^{7, 8} The MPA-induced GTP depletion of lymphocytes inhibits the induction of cyclin D3, a major component of cyclin-dependent kinase, and decreases the degradation of p27, a cyclin-dependent kinase inhibitor, thus arresting the cell cycle in early-to-mid-G₁ phase.^{9,10} Mycophenolate mofetil is now used in the management of auto-immune disorders such as idiopathic thrombocytopenic purpura, systemic lupus erythematosus, scleroderma and pemphigus vulgaris with success for some patients.¹¹ Thus it is now recommended that plasma mycophenolic acid concentrations should be monitored with the aim of achieving adequate immunosuppression with limited adverse effects.¹²

Since its discovery in 1970, and introduction into clinical practice in 1978, cyclosporin has become the most important immunosuppressive drug used to prevent organ transplant rejection. This has been achieved by virtue of the improved graft survival rates and adverse effect profiles in patients when

compared with that of the older agents. Cyclosporin has a complex pharmacokinetic profile with poor absorption, extensive metabolism to more than 30 metabolites and considerable inter- and intra-patient variability.¹³

Most effects of CsA are exerted through the formation of a specific complex with Cyclophilins (CYPS). The CYPS:CsA complex formed in the cytoplasm binds to calcineurin and inhibits its serine-threonine protein phosphatase activity, which represents a specific bottleneck of antigen-receptor signaling in immunocompetent cells. This leads to inactivation of lymphokine genes essential for T cell proliferation and activation, ultimately resulting in immunosuppression.¹⁴

Tacrolimus (FK-506) is used in organ transplantation because it promotes neurite outgrowth in vitro and enhances regeneration in peripheral nerve injury transection models.¹⁵ The immunosuppressive mechanism of FK-506 relates to its binding to the immunophilin FK-506-binding protein-12, inhibiting calcineurin. Inhibition of calcineurin phosphatase activity prevents translocation of nuclear factor of activated T cells to the cell nucleus, where it normally induces interleukin-2 secretion. Thus, the activation of T-cell proliferation by interleukin-2 is inhibited, preventing the normal cellular immune response.³

The aim of this study was to evaluate the effect of commonly used immunosuppressants; MPA, Cyclosporin and FK-506 on the proliferation and thus function of Human umbilical cord-derived endothelial cells (HUVECs) and Human adipose-derived adult stromal (ADAS) cells in vitro.

Materials and Methods

Cells: HUVECs and ADAS cells were obtained from the Comprehensive Cancer Center Cell Culture core facility, Duke University at passages 3 and 2 respectively. Since no patient identifiers of the original pathology specimens were recorded, the IRB declared this study exempt from full protocol review and from requirement of patient consent, consistent with Title 45 CFR Part 46, Subpart A, section b4

(<http://www.hhs.gov/ohrp/humansubjects/guidance/45cfr46.htm#46.101> section b.4.).

Cell Culture: HUVECs were cultured using 75 cm² flasks at a density of 2500/ cm² in EGM-MV BulletKit medium (Comprehensive Cancer Center Cell Culture core facility, Duke University, Durham, NC) containing 5% FBS, 12 µg/ml bovine brain extract, 10 ng/ml human epidermal growth factor, 1 µg/ml hydrocortisone and 1 µg/ml GA-1000 (gentamycin, amphotericin-B). Cells at passage 4–7 were used for the experiments. Cells were incubated in a humidified incubator at 37°C and 5% CO₂.

ADAS cells were cultured using 75 cm² flasks at a density of 2500/ cm² in preadipocyte medium (DMEM/Ham's F-12) (Comprehensive Cancer Center Cell Culture core facility, Duke University, Durham, NC) containing 5 µg/ml HEPES, 50 µg/ml fetal bovine serum, 5 µg/ml penicillin-streptomycin and 5 µg/ml amphotericin B. Cells at passage 3-7 were used for the experiments. Cells were incubated in a humidified incubator at 37°C and 5% CO₂.

Immunosuppressive Drugs: To evaluate the effects of immunosuppressive drugs on the proliferation of HUVECs, the cells were detached after reaching subconfluency with 0.25% trypsin-EDTA for 2 minutes. Then cells were seeded into a 96-well plate at a density of 2,500 cells per well. Using a triplicate of 3 wells, cells were either cultured using EGM-MV BulletKit medium alone, or treated with FK506 5mg/1ml (Astellas Pharma US, Inc., Deerfield, IL) at concentrations of 10, 100, 1,000, 10,000, and 100,000 ng/ml, or with CsA 250mg/5ml (Bedford Labs, Bedford, OH) at concentrations of 10, 100, 1,000, 10,000, 100,000 ng/ml and MPA 50 mg powder (Sigma-Aldrich, St. Louis, MO) at concentrations 5, 50, 500, 5,000, 50,000 ng/ml. After 5 days of incubation, the cell medium was removed and the plates were stored at -70°C. Cell proliferation was measured with the CyQUANT cell proliferation assay kit (SIGMA-ALDRICH, St. Louis, MO).

To evaluate the effects of immunosuppressive drugs on the proliferation of ADAS cells, the cells were detached with 0.25% trypsin-EDTA for 2 minutes after reaching subconfluency. Then cells were seeded into a 96-well plate at a density of 2,500 cells per well. Using a triplicate of 3 wells, cells were either cultured using preadipocyte medium (DMEM/Ham's F-12) alone, or treated with FK506 at concentrations of 10, 100, 1,000,

10,000, 100,000 ng/ml; CsA at concentrations of 10, 100, 1,000, 10,000, 100,000 ng/ml and MPA at concentrations of 5, 50, 500, 5,000, 50,000 ng/ml. After 5 days of incubation, the cell medium was removed and the plates were stored at -70°C. Cell proliferation was measured with the CyQUANT cell proliferation assay kit (Invitrogen-Molecular Probes, Eugene, OR).

The CyQUANT Cell Proliferation Assay Kit was used to quantify cell proliferation according to the manufacturer's specifications. The CyQUANT cell proliferation assay is a sensitive fluorescence-based microplate assay which utilizes CyQUANT GR dye for determining cell numbers. The assay yields a large fluorescence enhancement on binding to nucleic acids which can be measured using standard fluorescein excitation and emission wavelengths. The fluorescence emission of the dye-nucleic acid complexes correlates linearly with the cell number.¹⁶

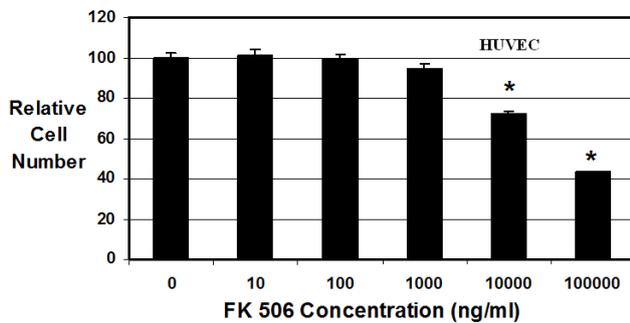
Briefly, cells were frozen, thawed, and lysed with the addition of the lysis buffer containing the green fluorescent dye, CyQUANT GR (Invitrogen-Molecular Probes, Eugene, OR), which binds to nucleic acids. The fluorescence levels were read on a fluorescent microplate reader (*fmax*; Molecular Devices, Sunnyvale, CA) with filters for 485 nm excitation and 538 nm emission.

Statistical Analysis: All experiments were performed three times, with results expressed as mean ± SEM. Differences among groups were analyzed by Sigma Stat (Systat Software, Inc., San Jose, CA) using a one-way analysis of variance with the Holm-Sidak method of multiple comparisons. A p value < 0.05 was considered necessary to indicate statistically significant differences among experimental groups.

Results

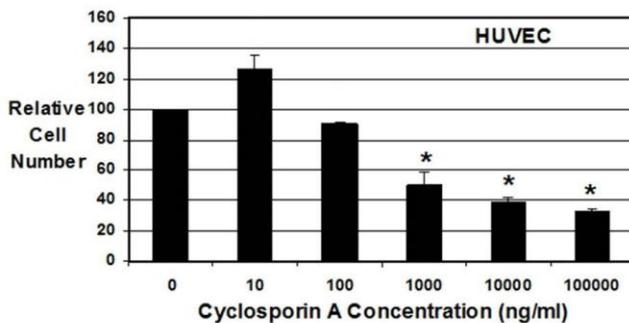
Proliferation of HUVEC cells exposed to drugs: All three drugs inhibited the proliferation of HUVEC in a dose-dependent manner. The lowest concentration of FK 506 to significantly inhibit the proliferation of HUVEC was 10,000 ng/ml ($p=0.023$) (Fig 1). The lowest concentration of CsA to significantly inhibit the proliferation of HUVEC was 1000 ng/ml ($p=0.003$) (Fig 2). The lowest concentration of

MPA to significantly inhibit the concentration of HUVEC was 500 ng/ml ($p=0.036$) (Fig 3).



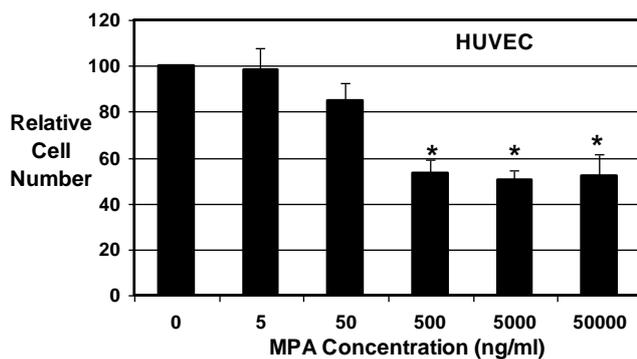
* $p < 0.05$ versus control (0 ng/ml)

Figure 1. Effects of FK 506 on HUVEC proliferation.



* $p < 0.05$ versus control (0 ng/ml)

Figure 2. Effects of CsA on HUVEC proliferation.

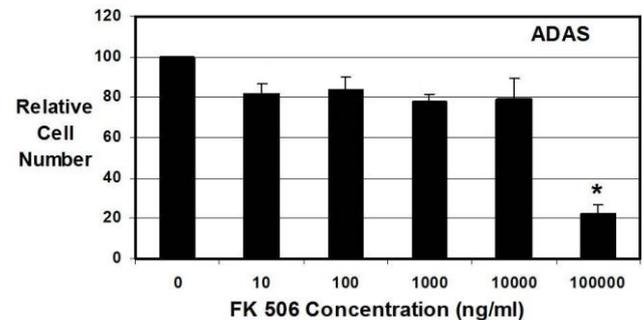


* $p < 0.05$ versus control (0 ng/ml)

Figure 3. Effects of MPA on HUVEC proliferation.

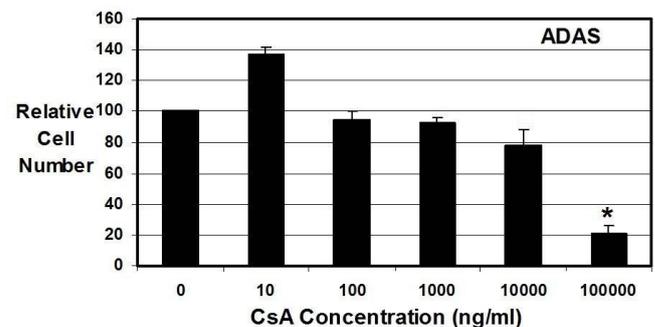
Proliferation of ADAS cells exposed to drugs: All the three drugs significantly inhibited the proliferation of ADAS cells in a dose-dependent manner. The lowest concentration of FK506 to significantly inhibit the proliferation of ADAS cells was 100,000 ng/ml ($p<0.001$) (Fig 4). The lowest concentration of CsA to inhibit the

proliferation of ADAS cells was 100,000 ng/ml ($p=0.001$) (Fig 5). The lowest concentration of MPA to inhibit the proliferation of ADAS cells was 5 ng/ml ($p=0.033$) (Fig 6).



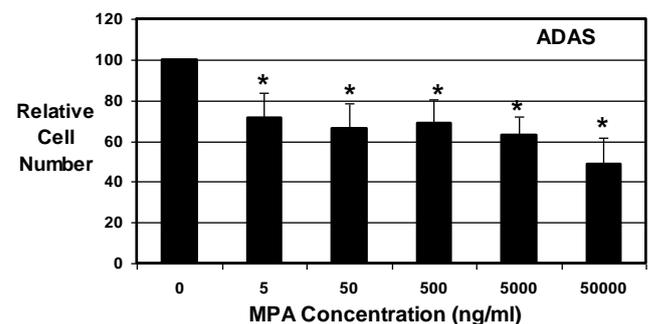
* $p < 0.05$ versus control (0 ng/ml)

Figure 4. Effects of FK 506 on ADAS cells proliferation.



* $p < 0.05$ versus control (0 ng/ml)

Figure 5. Effects of CsA on ADAS cells proliferation.



* $p < 0.05$ versus control (0 ng/ml)

Figure 6. Effects of MPA on ADAS cells proliferation.

Discussion

ADAS represent multi-potent cells which have the capacity to differentiate into an osteogenic lineage, and they may be used to heal defects of the craniofacial nerve injuries.

ADAS can ossify critical sized mouse calvarial defects without the need for pre-differentiation.¹⁷ Human Umbilical cord represents an attractive source for mesenchymal stem cells and endothelial cells which are both needed in bone regeneration. HUVEC have an imperative role in the complex process of angiogenesis and wound healing.¹⁸ Both ADAS and HUVEC are critical to the mechanism of tissue regeneration. These cells could act as key regulators of immune tolerance and attractive candidates for a cell-based therapy.¹⁹ In this study we assessed the proliferation of ADAS and HUVEC under different concentrations of immunosuppressive drugs.

Immunosuppressive drugs have been associated with side-effects and toxicities that limit their clinical utility. This is due, at least in part, to the fact that these drugs disrupt the function of many different cell types. Thus, elucidating the molecular mechanisms of these drugs could be important for designing specific immunosuppressants with no, or low, side-effects and toxicities.¹⁶ Recently it has been suggested that in order to optimize the therapeutic drug monitoring process of immunosuppressant drugs in different laboratories, there should be proper standardization of the laboratory procedures, introduction of global reference materials and strict compliance with internationally accepted laboratory guidelines.^{4,5}

CsA and FK506 exert their immunosuppressive effect by blocking the production of interleukin-2 (IL-2) by T helper cells, while MPA exerts its immunosuppressive effect by inhibiting proliferation of lymphocytes through the activation of a caspase-independent apoptotic signal.^{9,20} In their study of the *in vitro* effects of cyclosporin A (CsA), FK506 (tacrolimus), and rapamycin, on cultured human coronary artery smooth muscle cells, Hafizi et al²¹ found that CsA inhibited both platelet-derived growth factor (PDGF)-stimulated DNA synthesis and serum-induced proliferation at high concentrations ($\geq 1,000$ ng/ml). Another study has compared the systemic side effects of rapamycin after local and systemic treatment. This study showed that systemic rapamycin treatment caused more serious side effects than local treatment.²² Interestingly; FK506 antagonizes the antiproliferative properties of rapamycin on vascular SMCs because both agents bind to the same cytosolic receptor, FKBP12.²³

Mohacsi PJ et al²⁴ investigated "the different inhibitory effects of immunosuppressants on human and rat aortic smooth muscle and endothelial cell proliferation stimulated by endothelial cell growth factor or platelet-derived growth factor." They found that both rapamycin and mycophenolic acid were potent in inhibiting smooth muscle and endothelial cell proliferation. Cyclosporin demonstrated some inhibition of smooth muscle and endothelial cell proliferation, but the inhibitory concentration (IC50) was just below toxic levels. FK506 revealed a moderate inhibitory activity but, interestingly, only for human cells. They concluded that rapamycin was by far the most potent drug in having antiproliferative properties on vascular cells.

In the current study, all the three immunosuppressive agents inhibited ADAS cell and HUVEC proliferation in a dose-dependent manner *in vitro*. Our results showed that the minimum concentration of CsA to inhibit HUVEC proliferation was 1000 ng/ml. The minimum concentrations of FK506 and MPA to inhibit HUVEC proliferation were 10,000 ng/ml and 500 ng/ml, respectively. To elucidate the mechanisms involved in drug-induced cholestasis, a similar *in vitro* study on hepatic cells showed that at the same concentration (50 μ M), CsA induced more detrimental effects on hepatic cells than FK506 where the cellular efflux and uptake activities were enhanced and became irreversible, cellular components were disorganized and bile canaliculi were constricted.²⁵ Another study have also demonstrated that CsA exerts strong effects on the angiogenic potentiality of human microvascular endothelial cells, differentially prohibiting several stages in the *in vitro* angiogenic process. It was suggested that CsA suppresses the capacity of microvascular endothelial cells to undergo angiogenesis, inhibiting vascular homeostasis and inducing the vasculopathy associated with many immunological diseases as well as chronic rejection.²⁶

In our study MPA had the strongest inhibiting effect on ADAS cells *in vitro*. The minimum concentration of MPA to inhibit ADAS cell proliferation was 5 ng/ml. The minimum concentrations of FK506 and CsA to inhibit ADAS cell proliferation were 100,000 ng/ml and 100,000 ng/ml, respectively. FK506 and CsA inhibitory concentrations on both HUVEC and

ADAS cells were several orders of magnitude greater than those required for T cell inhibition.

Studies showed that FK506 inhibited inflammatory cytokine production from CD14+ monocytes as well as from T cells at concentrations less than 1 ng/ml, however, further studies of the pharmacokinetic, pharmacodynamic, and genetic characters of this drug are needed in order to avoid serious side effects.²⁷ CsA significantly enhanced the suppressive activity of regulatory T cells obtained from healthy humans at a concentration of 40 ng/ml by strongly increasing a protein involved in immune system responses (FoxP3) cell content.²⁸ Also the inhibitory concentration of MPA on lymphocyte function in vitro was 0.14 mg/l,⁸ which is higher than the concentrations required in our study to inhibit ADAS cells, but one-third of our observed inhibitory concentration for HUVEC.

In vitro studies had shown that the serum trough levels of an immunosuppressive dose of FK506 1.0 mg/kg taken orally ranged from 0.1 to 0.4 ng/ml²⁹ while those of Cyclosporin were 98 ng/ml.²⁸ Mean trough blood levels of MPA ranged between 69 and 340 ng/ml with a median of 147 ng/ml.⁸

The mean clearance of intravenously administered cyclosporin is 5.1 ± 1.5 ml/min/kg⁵ while the mean clearance of MPA is 59 ml/min (range 23–78 ml/min).⁸ Thus, the higher clearance of MPA means its effect will likely be of shorter duration compared to FK506 or CsA.

Our results showing that MPA had the strongest inhibitory effect on ADAS cells are in accordance to a previous study which showed that MPA had a strong inhibitory effect on human islet neogenesis.³⁰ Our results are also in accordance to another study which performed a meta-analysis to compare the safety of MPA with Mizoribine following renal transplantation, and found that recipients taking MPA had significantly more episodes of leucocytopenia, gastrointestinal disorders, cytomegalovirus infection and hepatic dysfunction. They recommended the use of Mizoribine as an alternative to MPA due to better efficacy and safety.³¹

Conclusions

In conclusion, the results of the present study indicated that all the three drugs inhibited the proliferation of ADAS and HUVEC cells in a

dose-dependent manner. MPA had the strongest inhibitory effect on HUVECs and ADAS cells. The FK506 and CsA minimum inhibitory concentrations on both HUVEC and ADAS cells were several orders of magnitude greater than those required for T cell inhibition and, therefore, dosages given to patients would not be expected to impair HUVEC or ADAS proliferation. On the other hand, the minimum inhibitory concentration of MPA on ADAS was lower than that observed in vitro for lymphocyte function and, therefore, could be of concern for ADAS and its relative applications in tissue engineering.

Acknowledgements

This study was supported, in part, by a grant from the Plastic Surgery Educational Foundation. The author wants to express his sincere gratitude to PhD Sausan Al Kawas and PhD Natheer Al Rawi for their assistance in writing the manuscript.

Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

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