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Effect of cyclosporine, tacrolimus and sirolimus on cellular senescence in renal epithelial cells

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ABSTRACT

Introduction: In transplantation medicine calcineurin inhibitors (CNI) still represent the backbone of immunosuppressive therapy. The nephrotoxic potential of the CNI Cyclosporine A (CsA) and Tacrolimus (FK506) is well recognized and CNI not only have been linked with toxicity, but also with cellular senescence which hinders parenchymal tissue regeneration and thus may prime kidneys for subsequent insults. To minimize pathological effects on kidney grafts, alternative immunosuppressive agents like mTOR inhibitors or the T-cell co-stimulation blocker Belatacept have been introduced.

Methods: We compared the effects of CsA, FK506 and Sirolimus on the process of cellular senescence in different human renal tubule cell types (HK2, RPTEC). Telomere length (by real time PCR), DNA synthesis (by BrdU incorporation), cell viability (by Resazurin conversion), gene expression (by RT-PCR), protein (by western blotting), Immuncytochemistry and H_2O_2 production (by Amplex Red® conversion) were evaluated.

Results: DNA synthesis was significantly reduced when cells were treated with cyclosporine but not with tacrolimus and sirolimus. Resazurin conversion was not altered by all three immunosuppressive agents. The gene expression as well as protein production of the cell cycle inhibitor p21 (CDKN1A) but not p16 (CDKN2A) was significantly induced by cyclosporine compared to the other two immunosuppressive agents when determined by western blotting an immuncytochemistry. Relative telomere length was reduced and hydrogen peroxide production increased after treatment with CsA but not with FK506 or sirolimus.

Conclusion: In summary, renal tubule cells exposed to CsA show clear signs of cellular senescence where on the contrary the second calcineurin inhibitor FK506 and the mTOR inhibitor sirolimus are not involved in such mechanisms. Chronic renal allograft dysfunction could be in part triggered by cellular senescence induced by immunosuppressive medication and the choice of drug could therefore influence long term outcome. Tacrolimus and Sirolimus are equally effective in avoiding cellular senescence compared to cyclosporine at least in parts due to a lack of induction of reactive oxygen species.

1. Introduction

The hallmark of transplantation medicine is the effective and safe use of immunosuppressive drugs to avoid allograft rejection. Since the introduction of calcineurin inhibitors in transplantation medicine they led to a significant reduction of acute allograft rejection and therefore improved long term graft survival. On the other hand, immunosuppressive agents lead to cellular changes mimicking chronic allograft nephropathy which leads to a loss in renal function and has been redefined by the two phenomenons interstitial fibrosis and tubular atrophy (Bennett et al., 1996). Several potential origins have been discussed including apoptosis or epithelial to mesenchymal transition triggering inflammation and vascular dysfunctions each hinder parenchymal tissue regeneration and thus prime kidneys for subsequent insults (Dempsey et al., 1998; Healy et al., 1998; Sosa Pena et al., 2016). For cyclosporine we have already shown a clear mechanism of cellular senescence in renal proximal tubule cells at least in parts triggered by the production of hydrogen peroxide (H2O2) (Jennings et al., 2007).

The paradoxon that calcineurin inhibitors are of need to prevent

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immunological rejection episodes but also lead to cellular impairment resulted in the development of novel immunosuppressive drugs like the mTOR inhibitor and the co-simulation blocker belatacept (Halloran, 2004). Since they are often of lower efficacy or have potential risk of side effects most patients after renal transplantation are still treated initially with one of the two CNIs.

Cellular senescence is described as the major cause of aging due to multifactorial growth arrest of cells. The mechanism of cellular senescence was initially described by Hayflick and Moorhead in 1961, who demonstrated that cultured human diploid fibroblasts proliferate only for a finite number of population doublings (Hayflick and Moorhead, 1961). Reaching a certain point, the cells arrest in the G1 phase of the cell division cycle. In contrast to apoptosis or cell death these cells remain metabolically active. Several mechanisms are involved in renal replicative senescence and have been described concisely and for the first time by Melk in 2003 (Melk, 2003).To protect the end of chromosomes of erosion they are capped by telomeres, tandemly repeated hexamers which work as the cellular replicative clock. Telomeres are shortened by each cell division, and once a critical telomere length has been reached the cell enters the state of replicative senescence to avoid inaccurate transcription of chromosomes (Harley, 1991).

Besides telomeres the cell cycle inhibitors p16 (CDKN2A) and p21 (CDKN1A) play a major role in the mechanisms of cellular senescence. While CDKN1A induction is directly related to telomere shortening via the p53 pathway, CDKN2A has been shown to induce cellular senescence independently of telomere attrition.

In the present study, we compare the influence of the three most widely used immunosuppressive agents in renal transplantation and their role in renal aging on two cell culture models. They have been chosen due to their differing mechanism of immortalization. Whereas in the HK2 cells the CDKN2A pathway is altered by Human Papilloma Virus transfection to guarantee further cellular propagation RPTECs possess a telomerase induction pathway to extend telomeres shortened due to cellular division (Ryan et al., 1994; Wieser et al., 2008). Primary cells have been evaluated in earlier studies but are not applicable for the investigation of replicative senescence due to a profoundly increased aging process by just by cellular division itself.

2. Methods

2.1. Cell culture

HK-2 cell line was purchased from American Type Culture Collection (ATCC no. CRL-2190). RPTEC/TERT1 human proximal tubule epithelial cell line was purchased from Evercyte (Vienna, Austria). Cells were routinely cultured on 10-cm culture dishes from Sarstedt (Nümbrecht, Germany). The culture medium was a 1:1 mixture of DMEM (Gibco 11,966) and Ham's F12 (Gibco 21,765) with a final concentration of 5 mM glucose, supplemented with 10 ng/ml human recombinant EGF (Sigma-Aldrich E9644), 36 ng/ml hydrocortisone (Sigma-Aldrich G0135), 5 µg/ml bovine insulin, 5 µg/ml human transferrin, 5 ng/ml sodium selenite (ITS; Sigma-Aldrich I1884), 2 mM L-alanyl-L-glutamine (Glutamax(R); Gibco 35,050), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich P4333). Cells were fed three times weekly and subcultivated by trypsinization when near confluence. HK-2 cells were used between passages 12 and 30, RPTEC-cells between passages 75 to 100.

Cells were trypsinized and seeded onto 6-well plates, glass coverslips on 12-well plates, or 96-well plates depending on the experiment. (1800-, 800-, and 100-µl medium volumes were used, respectively.) Cells were grown to full confluence before treatment for all experiments (unless otherwise stated) to best represent the in-vivo situation of a differentiated epithelial monolayer. CsA, FK506 and Sirolimus was dissolved in absolute ethanol to 10 mM, aliquoted, and frozen at − 20 °C. This stock was further diluted in absolute ethanol directly before treatment and added to the growth medium. Ethanol concentrations were at a maximum of 0.2% in all treatment groups, including controls.

RPTEC cells were used for all experiments except the determination of relative telomere length. Exposure time was 24 h in each experiment. Experiments for H2O2 production, Resazurin conversion and BrdU incorporation were carried out using 0, 0.5, 1, 2,5 and 10 µM of each substance. In all other experiments we used solely a dose of 10 µM each.

2.2. H_2O_2 production

Thirty microliters of freshly collected supernatants were added to 30 µl of 0.16 mM Amplex Red Ultra (Molecular Probes) and 1 U/ml horseradish peroxidase (HRP) in PBS. The plates were incubated for 1 h at 37 °C. Fluorescence was measured at 540-nm excitation and 595-nm emission. Relative fluorescent units (RFU) were converted to fold over basal values.

2.3. Determination of viable cell number and DNA synthesis

Cells were treated on 96-well plates and incubated for 2 h with 44 µM resazurin and 10 µM bromodeoxyuridine (BrdU)-labeling reagent (Cell Proliferation ELISA, Roche) in growth medium. Resazurin reduced to resorufin was measured at 540 nm excitation and 590 nm emission. Resazurin reduction is similar to the MTT assay and is directly proportional to the number of viable cells (Liu, 1981). For subsequent determination of BrdU incorporation, cells were carefully washed with PBS to avoid interference with the resazurin assay before fixing them in FixDenat solution (Roche) for 30 min. Cells were then incubated for 1 h with anti-BrdU HRP-conjugated Fab fragments (Roche) with 300-rpm orbital shaking. Cells were washed and incubated for 30 min in 20 µM Amplex Red Ultra (Molecular Probes), 5 mM H_2O_2 in PBS for colorimetric quantification of BrdU. Amplex red is converted to resorufin in the presence of H_2O_2 and HRP. Since HRP is limited in this reaction, fluorescence is directly proportional to antibody bound and thus to BrdU incorporated. Fluorescence was measured at 540 nm excitation and 595 nm emission. RFU were converted to % control values.

2.4. Quantitative real-time PCR

Total RNA was isolated using standard phenol/chloroform-based techniques (Tri Reagent, Sigma). RNA was quantified photometrically and 1 µg of RNA was transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen), nucleotides (Amersham, Buckinghamshire, UK), and random hexamers (Roche). The cDNA equivalent of 50 ng RNA was used in a qPCR reaction mix of 20 µl, consisting of 2 × TaqMan® Universal PCR Master Mix and pre-designed RT-qPCR assays for p21 (p21 WAF1, CDKN1A) and p16 (p16INK4a, CDKN2A) (Taqman Hs00355782_m1; Hs00923894_m1; ThermoFisher Scientific), 18 s RNA (Applied Biosystems, 4310893E) served as a standard reference gene. Experiments were carried out using the Applied Biosystems 7500 Fast RT qPCR System. A thermal cycling profile started with 2 min at 50 °C (RNAse inhibitor activation) and 10 min at 95 °C to activate polymerase. Repeating cycles were performed 40 times at 95 °C for 15 s, followed by 60 °C for 1 min. Samples were run in duplicate, and the gene expression levels were calculated using the ΔΔCt method.

2.5. Telomere length assay

Genomic DNA was isolated from cell cultures with DNeasy Blood & Tissue Kit (Qiagen) and quantified using the fluorescent-based Picogreen assay (Molecular Probes). Relative telomere length was determined using an optimized assay originally described by Cawthon (Cawthon, 2002; Koppelstaetter et al., 2005). Separate PCR experiments were performed for telomere (T) and 36B4, a single-copy gene (S), in 96-well optical reaction plates (Applied Biosystems). Twenty