The immunosuppressive drug mycophenolic acid reduces endothelin-1 synthesis in endothelial cells and renal epithelial cells

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ABSTRACT

Several studies have demonstrated that endothelin-1 (ET-1) plays an important pathophysiological role in ischaemic renal failure and drug-induced renal injury, such as cyclosporine A (CsA)- and tacrolimus-associated nephrotoxicity. This study aimed to investigate whether the new immunosuppressive drug mycophenolic acid (MPA), which in contrast with CsA and tacrolimus lacks nephrotoxic side effects, modulates ET-1 synthesis in endothelial cells and renal epithelial cells. ET-1 release by cultured human umbilical vein endothelial cells (HUVEC), human renal artery endothelial cells (RAEC) and rabbit proximal tubule cells was measured with a specific ELISA. ET-1 mRNA expression was investigated by reverse transcription–PCR. MPA (2.5–50 μg/ml) induced a significant decrease in ET-1 mRNA expression (minimum 51.8 ± 3.8% of control; \( P < 0.001 \)) in HUVEC and RAEC. After a 48 h incubation with MPA (1–50 μg/ml), a significant decrease in ET-1 release per culture well (minimum 56.8 ± 1.7%; \( P < 0.001 \)) and DNA content per culture well (minimum 58.7 ± 1.9%; \( P < 0.001 \)) was observed with HUVEC and RAEC, whereas ET-1 release referred to the DNA content in the corresponding culture well did not differ significantly from controls. In rabbit proximal tubule cells, ET-1 release referred to the cell number in the corresponding culture well was also reduced after incubation with MPA (minimum 86.2 ± 2.4%; \( P < 0.05 \)). This study provides evidence that, in contrast with CsA and tacrolimus, MPA does not stimulate ET-1 synthesis. The present results might explain the clinical observation that renal function often improves when CsA or tacrolimus is replaced by mycophenolate mofetil.

INTRODUCTION

The vasoconstrictive peptide endothelin-1 (ET-1) seems to be an important mediator of ischaemic renal failure and drug-induced renal injury, such as cyclosporine A (CsA)- and tacrolimus-associated nephrotoxicity [1–4]. Both drugs have been shown to stimulate ET-1 synthesis in endothelial cells and renal epithelial cells [1,5–9]. Mycophenolate mofetil (MMF) is a new immunosuppressive drug which is converted in vivo into mycophenolic acid (MPA) [10]. MMF does not seem to have nephrotoxic side effects, and recently published studies have demonstrated that renal function was markedly improved when the immunosuppressive therapy was switched from CsA or tacrolimus to MMF, or when MMF was added and CsA or tacrolimus doses were...
reduced [11–15]. The present study aimed to investigate the effect of MPA on ET-1 synthesis in endothelial cells and renal epithelial cells.

MATERIALS AND METHODS

Cell culture

Human umbilical vein endothelial cells (HUVEC) and human renal artery endothelial cells (RAEC), purchased from Clonetics, were cultured in EGM-2 medium (Clonetics; 2% (v/v) fetal calf serum (FCS)) and EGM-2-MV medium (5% FCS) respectively. For investigation of ET-1 release and ET-1 mRNA expression, HUVEC (passages 3–4) and RAEC (passages 5–6) were seeded into 24-well plates and 6-well plates respectively, and experiments were performed with confluent monolayers in culture medium containing 1% FCS (PAA Laboratories). The bipolar differentiated rabbit proximal tubule cell line PT-1 was cultured as described previously [6], and for investigation of ET-1 release cells were seeded into 6-well plates in culture medium (Dulbecco’s modified Eagle’s medium/Ham’s F-12, 1:1, v/v; Biochrom) supplemented with 1% FCS.

For investigation of ET-1 release into the cell culture supernatant, cells (HUVEC, passage 4; RAEC, passage 5) were incubated for 48 h with MPA (1–50 μg/ml; Sigma) or vehicle. The effect of MPA on ET-1 mRNA expression was investigated by incubating HUVEC (passage 3) for 3, 4 or 5 h and RAEC (passage 6) for 3.5 h with 2.5, 10, 25 or 50 μg/ml MPA.

Measurement of ET-1 release

ET-1 was measured with a specific ELISA kit (Biotrend). Cross-reactivity was as follows: ET-1, 100%; ET-2, 3.3%; ET-3, < 0.1%; Big ET-1, < 0.1%; Big ET-3, < 0.1%. Intra-assay coefficients of variation (n = 24) were 2.9% (9.7 pg/ml) and 2.1% (56.2 pg/ml); inter-assay coefficients of variation (n = 8) were 2.9% (10.0 pg/ml) and 3.3% (55.4 pg/ml). No immunoreactive ET-1 was detected in cell culture medium that had not been incubated with the cells.

Measurement of DNA and cell number

The DNA content of cells was measured as described previously [16] by fluorescent DNA staining with bisbenzimide (Sigma) using calf thymus DNA as a standard. Cell numbers were counted with a cell counter (Cell-Dyn 3500; Abbott).

RNA isolation and reverse transcription–PCR (RT-PCR) of ET-1 mRNA

ET-1 mRNA expression was investigated by RT-PCR. Total RNA was extracted with a High Pure RNA Isolation Kit which includes DNA digestion (Roche Diagnostics). For RT-PCR the following oligonucleotide primers were used: preproET-1 [17] from nucleotide +415 to +662 (a 248 bp fragment; accession no. Y00749): sense primer, 5′-TGCTCTCCCTGATGGATA-3′; antisense primer, 5′-TTCTCCATAATGTCTTTCAGCC-3′ (exon 2–exon 4); β-actin [18] from nucleotide +144 to +683 (a 540 bp fragment; accession no. M10277): sense primer, 5′-GTGGGGGCCCCTAGGCCC-3′; antisense primer, 5′-CTCCTTAATGTCACGCAGATTTC-3′ (exon 2–exon 4). DNA amplification was performed using LightCycler technology (Idaho Technology; LightCycler–DNA Master SYBR Green I was from Roche Diagnostics). PCR products were quantified using the LightCycler software. ET-1 mRNA was referred to β-actin mRNA in the corresponding samples. No detectable PCR products were present in water controls or in controls amplified without prior reverse transcription. For visualization, PCR products were applied to 1% (w/v) agarose gels stained with GelStar (Biozym).

Determination of lactate dehydrogenase (LDH) release

LDH release into the cell culture supernatants was determined with a coupled enzymic assay (CytoTox 96® Non-Radioactive Cytotoxicity Assay; Promega).

Statistical analysis

Results represent means ± S.E.M. Differences between ET-1 synthesis were evaluated by one-way ANOVA, followed by the Newman–Keuls test.

RESULTS

Incubation of HUVEC and RAEC with the immunosuppressive substance MPA induced a significant decrease in ET-1 release and DNA content per well, whereas ET-1 release referred to the DNA content in the corresponding culture well did not differ significantly from controls (Figure 1). In both cell types, the level of ET-1 mRNA was significantly decreased after incubation with MPA (Figure 2). β-Actin mRNA was only slightly reduced in HUVEC, and was not altered significantly in RAEC. In rabbit proximal tubule cells, MPA induced a decrease in ET-1 release per well (minimum 80.4% ± 3.6% of control; n = 3), a slight decrease in cell number (minimum 87.7% ± 3.5%) and a moderate decrease in ET-1 release when referred to the corresponding cell number (Figure 3).

Measurement of LDH release as an indicator of cell cytotoxicity revealed that the applied MPA concentrations had no cytotoxic potential. Results were as follows. HUVEC: 85.3% ± 3.7% of control release (P < 0.01 compared with control), 73.9% ± 2.2% (P < 0.001), 75.9% ± 3.5% (P < 0.001), 74.2% ± 2.9% (P < 0.001),
DISCUSSION

Several studies have demonstrated that ET-1 is an important mediator of CsA- and tacrolimus-associated nephrotoxicity [1,19,20]. CsA and tacrolimus have been shown to stimulate ET-1 synthesis in endothelial cells, renal epithelial cells, smooth muscle cells and mesangial cells [1,5–9,21,22]. In addition, application of ET$_A$

71.9±2.1% ($P<0.001$), 72.6±3.5% ($P<0.001$) and 71.0±2.1% ($P<0.001$) with 1, 2.5, 5, 10, 15, 25 and 50 µg/ml MPA respectively; controls, 100±2.5% ($n=8$). RAEC: 73.8±2.2%, 72.6±1.3%, 68.2±1.4%, 69.3±2.3%, 65.0±2.9%, 71.7±3.8% and 61.7±2.8% with 1, 2.5, 5, 10, 15, 25 and 50 µg/ml MPA respectively (all $P<0.001$); controls, 100±1.3% ($n=8$). Renal proximal tubule cells: 61.7±2.2%, 62.4±2.3%, 65.4±2.5%, 62.7±1.0%, 64.4±2.1% and 66.9±2.4% with 2.5, 5, 10, 15, 25 and 50 µg/ml MPA respectively (all $P<0.001$); controls, 100±2.6% ($n=3$).
receptor antagonists attenuated CsA-induced renal hypoperfusion [1,23]. In the present study, we have demonstrated that the new immunosuppressive drug MPA, applied at concentrations in the range of therapeutic blood levels [15,24], significantly inhibited ET-1 mRNA expression in HUVEC and RAEC. In both cell types, incubation with MPA for 48 h induced a significant decrease in ET-1 release and a parallel decrease in DNA content, whereas ET-1 release referred to the DNA content in the corresponding culture well did not differ significantly from controls. In rabbit proximal tubule cells, ET-1 release referred to the corresponding cell number was significantly reduced after incubation with MPA. The discrepancy between ET-1 mRNA expression and ET-1 release, observed in endothelial cells, might be explained by different incubation periods for the investigation of mRNA expression (3–5 h) and of ET-1 release, observed in endothelial cells, to which ET-1 mRNA expression and ET-1 release were referred respectively.

MPA exerts its immunosuppressive effect by inhibition of IMP dehydrogenase, a key enzyme in de novo DNA synthesis [25]. Lymphocytes are more dependent on de novo DNA synthesis than other cell types; however, inhibitory effects of MPA on DNA synthesis in cultured endothelial cells and on renal tubular cell growth has also been observed in previous studies [26,27]. From in vitro studies it might be difficult to understand the net effect of long-term MPA application on in vivo ET-1 synthesis. However, in summary, our in vitro results suggest that MPA induces a slight to moderate decrease in ET-1 synthesis, or at least, in contrast with CsA and tacrolimus, does not stimulate ET-1 synthesis. These findings might explain the clinical experience, confirmed by several studies [10–15], that MPA, in contrast with CsA and tacrolimus, does not exert nephrotoxic side effects, and that switching immunosuppressive therapy from these substances to MPA, or addition of MPA and reduction of CsA or tacrolimus, is often accompanied by an improvement in renal function.

ACKNOWLEDGMENT

We thank Gisela Sailer for expert technical assistance.

REFERENCES


