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ORIGINAL ARTICLE

Effects of tacrolimus, cyclosporin A and sirolimus on MG63 cells

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Summary

The reduction in bone mineral density after organ transplantation results in increased morbidity (post-transplantation bone disease) and remains an unsolved problem. A connection with the long-term application of nonglucocorticoidal immunosuppressants is the subject of controversial discussion. We hypothesized that such substances have an influence on the skeletal system on the cellular level by modulating osteoblast differentiation. Therefore, we investigated the effects of tacrolimus, cyclosporin A and sirolimus as representative substances of nonglucocorticoidal immunosuppressants on cell proliferation and expression of bone tissue-specific genes of human osteoblasts (MG63). None of the examined substances affected cell proliferation, but all influenced the gene expression pattern towards change in cell differentiation. In detail, collagen III and XII, matrix metalloproteinase 2, SMAD2, epithelial growth factor receptor, annexin V and osteonectin expression were increased by all of the examined substances. Tacrolimus, cyclosporin A and sirolimus influence intracellular signalling pathways, transmembranous receptors and bone-specific matrix synthesis. They do not have antiproliferative or toxic effects. We postulate that the shown changes of osteoblast differentiation cause post-transplantation disease.

Introduction

Since the introduction of modern immunosuppressants, such as tacrolimus, cyclosporin A and sirolimus, in the field of organ transplantation, rejections have been drastically reduced and the survival rate of transplanted patients has been improved [1–3]. In contrast, morbidity due to so-called post-transplantation bone disease has not decreased. During the first year after transplantation of liver, kidney, heart or lung, a considerable decrease in bone mineral density (BMD) has been demonstrated [4–11]. This results in pain [12] and particularly in an incidence of spontaneous osteoporotic fractures as high as 25–65% [13,14]. Due to the reduced bone quality, a higher rate of complications such as prolonged immobilization and non-union is seen in these patients [15,16].

There is an ongoing controversial debate about whether modern immunosuppressants have a negative influence on bone metabolism, and therefore, can be partially held responsible for the development of post-transplantation bone disease. Results of investigations of various authors using different experimental models (animal, human, *in vitro*) are contradictory [17–20]. Therefore, results from animal experiments may not be transferred to humans.

Hence, besides *in vivo* studies in humans, the analysis of effects of immunosuppressants on human osteoblasts is useful and may establish a basis for new therapeutic approaches. The aim of this study was to analyse the effects of tacrolimus, cyclosporin A and sirolimus – modern agents for continuous immunosuppressive therapy after organ transplantation – independently of co-medication and underlying disease *in vitro*. We hypothesized an

influence of these substances on the skeletal system through changes of osteoblast differentiation and proliferation.

Materials and methods

Cell culture

The experiments were performed with the well-documented human osteoblast cell line MG63. The cells were seeded in 300-cm^2 culture flasks (Falcon, Greiner, Germany) at a density of 5000 cells/cm^2 and fed with medium [Hams-F12 + 10% fetal calf serum (FCS) + 100 IU/ml penicillin + 100 μg/ml streptomycin + 5 μg/ml amphotericin] (Sigma-Aldrich, Munich, Germany) at 2-day intervals.

Cell proliferation measurement

The cells were seeded nonconfluently (5000 cells/well into 48-multiwell plates) (Falcon). After 24 h, the medium was changed for the first time and the immunosuppressants were added in a therapeutic serum concentration (tacrolimus 10 ng/ml, cyclosporin A 200 ng/ml and sirolimus 12 ng/ml). Afterwards, medium and immunosuppressants were renewed every other day. Every 4 days, the cells were counted after trypsination with 200 μl of 0.25% trypsin per well in a CASY II cell counter (Schärfe System, Reutlingen, Germany) ($n = 12$ wells per timepoint and immunosuppressant).

RNA extraction and gene expression analysis

The gene expression of selected signal molecules [transforming growth factor (TGF)-β1 receptor, SMAD2, SMAD9, epidermal growth factor (EGF) receptor and annexin V] and matrix proteins [matrix metalloproteinase 2 (MMP2), biglycan, collagen III and XII, and osteonectin] was examined using confluent cells.

The MG63 cells were fed with medium containing immunosuppressants in the concentrations mentioned above every 2 days for 5 days. After extensive washing with phosphate-buffered saline, the cells were detached from the culture flasks with a cell scraper. Afterwards, the RNA was extracted with a Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) ($n = 4$ for each immunosuppressant). The integrity of the RNA was confirmed with a bioanalyzer (Eukaryote Total RNA Nano, Agilent Technologies 2100 Bioanalyzer; Agilent Technologies, Waldbronn, Germany) in a biosizing assay prior to further analysis.

As starting material 3 μg total RNA was used. The cDNA first-strand synthesis was performed at 37 °C for 25 min. The subsequent labelling was achieved with biotin-16-dUTP according to the distributor's instructions. This was followed by 30 cycles of polymerase chain reaction (PCR) amplification of the RNA for 1 min each at 85, 50 and 72 °C. The PCR product was hybridized at 56 °C for 12 h using Oligo GEArray® Human Osteogenesis Microarray (OHS-026; Superarray, Frederick, MD, USA). Prior to this, unspecific binding was blocked by prehybridization with salmon spermatic DNA (Roche Diagnostics, Mannheim, Germany) for 2 h. After labelling with streptavidin–biotin–antibody and CDP-star-solution (Biomol, Hamburg, Germany), the arrays were transferred to X-ray films (Kodak, Stuttgart, Germany) and exposure series were performed. The films were developed and scanned, and the gene expression was evaluated with the softwares ScanAlyze (Eisen Software, Berkeley, CA, USA) and GEArray Analyzer (Superarray Software).

Signal molecules

Multiple signalling pathways, including bone morphogenetic protein (BMP) and TGF-β are required for bone formation [21]. There are different types of SMADs (mothers against decapentaplegic-related proteins): transducing TGF-β/activin signals (e.g. SMAD2) [22] or transducing BMP-like signals and mediating the osteogenic effect of bone morphogenetic proteins (BMPs) (e.g. SMAD9) [23]. SMAD2 operates downstream of TGF-β. TGF-β is expressed in osteoblastic cells and acts as an autocrine as well as a paracrine factor in the regulation of osteoblastic functions [24]. The function of the TGF-β1 receptor is closely related to the SMADs that are responsible for intracellular signal transduction [25,26]. The synthesis of extracellular matrix (ECM), the proliferation of osteoblasts as well as osteoclasts are regulated together [27–29]. EGF receptor influences the remodelling of bone tissue through mediation of the differentiation of osteoblasts and osteoclasts [30]. Annexin V is involved in the mineralization of bone matrix through its regulation of the binding of calcium and phospholipids to the cytoskeleton and the extracellular collagen [31].

Matrix proteins

Matrix metalloproteinase-2 is responsible for the degradation of the ECM. It can lead to the degradation of different collagens (i.e. collagen I and IV) [32]. Biglycan as a proteoglycan is a crucial component of the ECM of bone tissue and is necessary for collagenic fibrillogenesis. Collagen III and XII are involved in the creation of the ECM structure. Osteonectin has an influence on mineralization of the ECM through the cross-linking of hydroxyapatite and collagens [33].

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Statistical analysis

Statistical analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). The signal values of the single genes were normalized after subtraction of the background noise versus the house-keeping gene GAPDH. For each gene mean and SD were calculated in each treatment group. The nonparametric Mann–Whitney *U*-test for independent variables was applied for analysis of significance. The level of significance was set at $P \leq 0.05$.

Results

Cell proliferation

All investigated groups showed a sigmoidal proliferation curve typical for monolayer cell culture. The growth was exponential up to day 4, followed by linear growth until day 8 and approximation of the maximum cell density at day 12 (Fig. 1). None of the examined substances influenced the osteoblast proliferation rate over the investigated period.

Gene expression analysis

Signal molecules

Tacrolimus, cyclosporin A and sirolimus led to a significant increase in the expression of SMAD2 [ninefold, ninefold and twofold compared with the control ($P < 0.05$)]. Tacrolimus and cyclosporin A enhanced the expression of the TGF-β1 receptor eight- and sevenfold in comparison with the control ($P < 0.05$), whereas sirolimus had no influence on this parameter.

The expression of SMAD9 showed contradictory behaviour. Whereas sirolimus did not influence it either, tacrolimus and cyclosporin A suppressed its expression almost entirely ($P < 0.05$). All three examined substances increased the expression of the EGF receptor: cyclosporin A sevenfold, tacrolimus fourfold and sirolimus threefold compared with the control group ($P < 0.05$).

The increase in the expression of annexin V was found to be sixfold for tacrolimus, ninefold for cyclosporin A and threefold for sirolimus ($P < 0.05$) (Fig. 2).

Matrix proteins

All analysed substances increased the expression of MMP2 – cyclosporin A fivefold, tacrolimus fourfold and sirolimus fivefold compared with the control group ($P < 0.05$). The gene expression of biglycan was raised twofold under tacrolimus in comparison with the control. In contrast, sirolimus suppressed the expression of biglycan 10-fold compared with controls ($P < 0.05$), and cyclosporin A had no influence on this parameter.

The expression of collagen types III and XII was increased under the influence of tacrolimus, cyclosporin A and sirolimus. Collagen type III was increased 4.5-, five-, and fourfold and collagen type XII 21-, 19-, and ninefold, respectively, in comparison with the controls ($P < 0.05$). Osteonectin expression was increased 21-fold under the influence of tacrolimus, 19-fold under cyclosporin A and ninefold under sirolimus ($P < 0.05$) (Fig. 3).

Discussion

A connection between long-term therapy with the immunosuppressants tacrolimus, cyclosporin A and sirolimus, which are used after organ transplantation, and post-transplantation bone disease is difficult to verify *in vivo*, due to the impossibility of differentiating transplant, co-medication, and immunosuppressant-associated side effects on BMD. On the other hand, species-specific

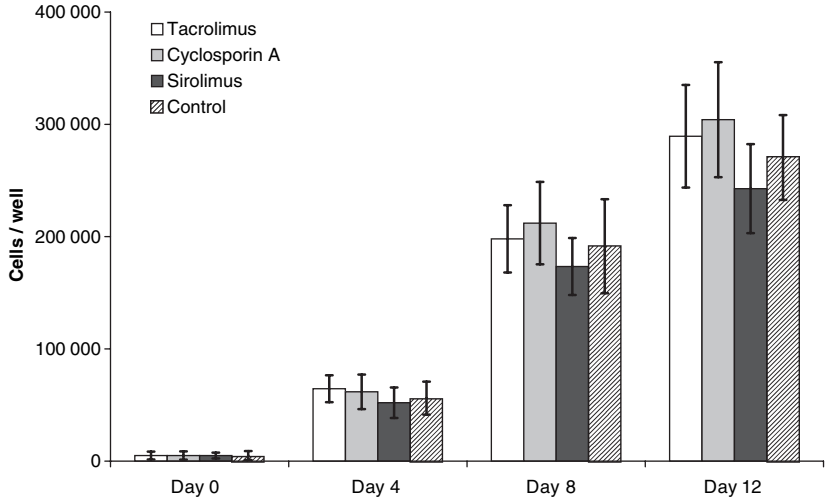

Figure 1 Cell counts of MG63 cells after incubation with tacrolimus, cyclosporin A and sirolimus (mean values and standard deviations). There were no significant differences between the immunosuppressants and the control group.

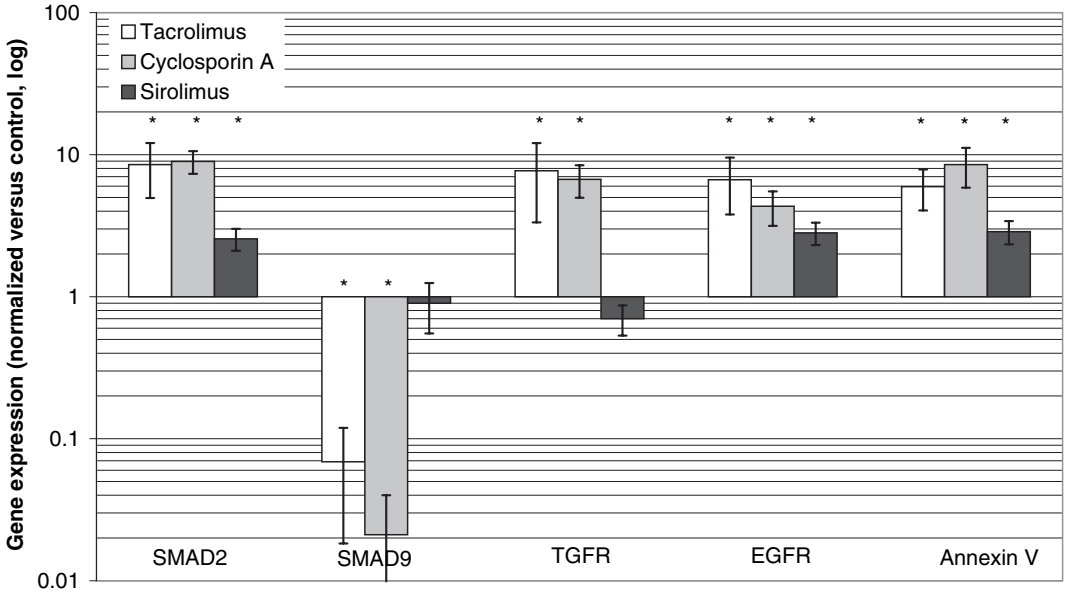

Figure 2 Gene expression profile of signal molecules analysed after 5 days therapy (logarithmic graphic representation, mean values and standard deviations, $*P < 0.05$).

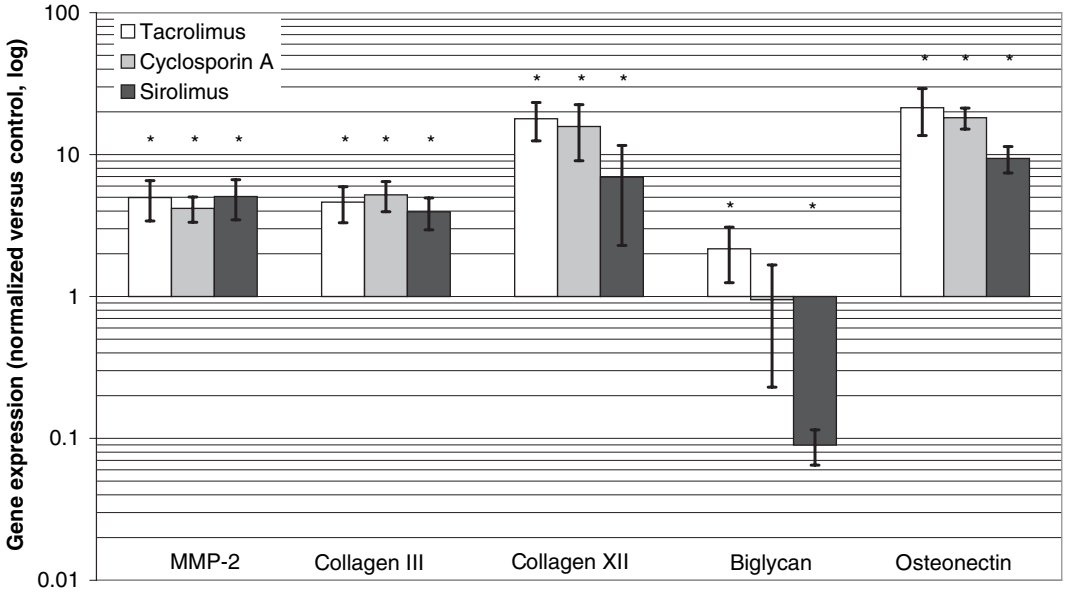

Figure 3 Gene expression profile of matrix molecules analysed after 5 days therapy (logarithmic graphic representation, mean values and standard deviations, $*P < 0.05$).

effects of the immunosuppressants are probable, due to their individual mechanisms of action. Therefore, a transfer of results from animal experiments seems problematic [34,35]. This study analysed the effects of tacrolimus, cyclosporin A and sirolimus for the first time independently of co-medications and underlying diseases *in vitro*, using the human osteoblast cell line MG63 [36,37].

Our results did not show any change in cell proliferation through any of the tested substances. This correlates with the findings of Romero *et al.*, who showed no negative influence of sirolimus on bone formation and remodelling in a rat model [38]. In contrast, other study groups have described a suppression of cell proliferation through cyclosporin A [39], tacrolimus and sirolimus [40], using

the rat osteoblast-line ROS. This contradiction may be explained by a species-specific effect of all tested immunosuppressants on osteoprogenitor cells.

This study was able to show for the first time the upregulation of MMP2 expression through tacrolimus, cyclosporin A and sirolimus in a comparable amount. So far, similar effects have been observed on different cells – e.g. mesangial cells and T lymphocytes [26,41]. There is strong evidence that MMP2 plays a crucial role during osteogenesis and bone remodelling through degradation of ECM proteins. If overexpressed, it leads to an increase in bone turnover, resulting in osteoporosis [42]. While del Pozo *et al.* found a constant BMD in rats when applying the same concentration of cyclosporin A as used in this study [17], other groups, using up to threefold higher concentrations, demonstrated the development of osteoporosis [18,43,44]. A concentration-dependent shift of the equilibrium of ECM formation and resorption through MMP2 may explain the differing results in humans.

The expression of collagen types III and XII was increased in virtually the same amount as the MMP2 expression, possibly pointing to an increased, but balanced turnover of ECM. Collagen types III and XII are a fibril-associated collagen adorning the surface of collagens and linking collagen fibrils to one another and to other components in the ECM [45,46]. Especially collagen type III is a molecule characteristic of an early stage of osteoblast differentiation [47].

Annexin V, a protein crucial for bone matrix calcification, was overexpressed by all of the tested substances [48]. Biglycan expression was suppressed by sirolimus and was not influenced by cyclosporin A. In contrast, tacrolimus overexpressed biglycan, an ECM protein that plays an important role in osteoblast differentiation. Biglycan-deficient mice develop age-related osteopenia [49]. The overexpression through tacrolimus correlates with observations of Yoshikawa *et al.* in rats, where tacrolimus therapy led to a higher osteogenic potential of bone marrow-derived cells [50]. Transferring these results to humans, an influence on osteoblast differentiation and function with increased osteogenesis and remodelling through tacrolimus can be postulated. This has been shown in different rat models – Kirino *et al.* [51] showed the occurrence of osteoporosis and Inoue *et al.* [20] could not find an influence on BMD.

Osteonectin expression was stimulated by all of the tested substances. Osteonectin binds to collagen and regulates angiogenesis, MMP expression and cell proliferation in bone tissue [33]. Thus, osteonectin-deficient mice show a reduced number of osteoblasts, and reduced and catabolic bone turnover, resulting in severe osteoporosis. These findings are consistent with the fact that osteonectin expression is reduced in osteogenesis imperfecta [52].

Osteoblast differentiation, proliferation and the expression of ECM proteins is controlled by the TGF-β and BMP signalling pathways, among others [53]. Tacrolimus and cyclosporin A enhanced the TGF-β signalling pathway by stimulating the expression of SMAD2 and TGF-β1 receptor expression. On the other hand, tacrolimus and cyclosporin A inhibited the BMP signalling pathway by suppressing the expression of SMAD9. In contrast, sirolimus had no effect on TGF-β1 receptor or SMAD9 expression.

The EGF receptor was overexpressed by all of the tested immunosuppressants. The EGF receptor is involved in the early stages of cellular proliferation and differentiation in mineralized tissues [54]. EGF receptor-deficient mice show a decrease in the number of osteoblasts and osteoclasts, resulting in delayed ossification [55].

None of the investigated immunosuppressants influenced the proliferation of human osteoblasts. Any antiproliferative or cytotoxic effect on human osteoblasts could thereby be excluded. This is contradictory to *in vitro* and *in vivo* experiments in animal models, which may be explained by the species-specific mechanism of action of these drugs. In all groups, an alteration in the expression of osteogenic genes, coding for signal transduction as well as for ECM, was shown. This implies a change in cellular differentiation.

In conclusion, long-term administration of immunosuppressants modulates bone tissue differentiation at the cellular as well as at the matrix level. Obviously, this modulation induces changes in the skeletal system, regardless of any co-medication or disease, resulting in and promoting post-transplantation bone disease. Our results do not provide suggest that any one of the investigated substances should be given preference over the others for the prevention of post-transplantation bone disease.

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