Conversion from cyclosporin A to tacrolimus is safe and decreases blood pressure, cholesterol levels and TGF-β1 type I receptor expression

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Abstract: To determine whether conversion from cyclosporin A (CsA) to tacrolimus (TAC)-based immunosuppressive therapy is safe and might lead to improvement in the clinical side effect profile we studied 55 cardiac allograft recipients. Ten stable patients were electively converted (0.2–1.5 yr after transplantation; group I) and 45 patients were converted on indication (0.5–14 yr after transplantation; group II). We studied blood pressure, cholesterol level and renal function in all patients. To unravel the mechanisms by which CsA may exert its toxic effects and to evaluate whether conversion is associated with immune activation, we analyzed the transforming growth factor (TGF)-β1 system and intragraft interleukin (IL)-2 and IL-15 mRNA expression by real-time reverse transcription-polymerase chain reaction (RT-PCR) and quantitative flow cytometry in the selectively converted patients (group I).
Conversion did not result in immune activation as no clinical, histological or molecular signs of immune activation (increased intragraft IL-2 and IL-15 messenger RNA (mRNA) expression) leading to rejection were found. It did not improve renal function neither in patient group I nor in patient group II. However, after conversion the blood pressure decreased (group I: systolic 154 ± 16 vs 143 ± 21 mmHg, $p = 0.03$, diastolic: 99 ± 11 vs 90 ± 11, $p = 0.02$ and group II: systolic 155 ± 17 vs 142 ± 14, $p < 0.001$, diastolic: 99 ± 11 vs 91 ± 8 mmHg, $p < 0.001$). Likewise, the cholesterol levels improved (group I: 6.6 ± 0.5 vs 5.7 ± 0.3 mmol/L, $p = 0.001$ and group II: 7.1 ± 1.7 vs 6.1 ± 1.7 mmol/L, $p = 0.001$). When patients were treated with TAC the ongoing rejections ($n = 4$) resolved and gum hyperplasia disappeared ($n = 5$). Conversion was associated with a two-fold lower TGF-β1 type I receptor expression on peripheral lymphocytes and monocytes ($p = 0.02$ and $p = 0.002$, respectively).
Conversion from CsA to TAC results in improvement of blood pressure and cholesterol levels and does not induce immune activation. These beneficial effects were accompanied with lower TGF-β1 type I receptor expression.

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After the introduction of cyclosporin A (CsA) the results of clinical heart transplantation significantly improved. Therefore, most immunosuppressive regimens are CsA based (1). CsA affects T-cell function at the level of messenger RNA (mRNA) transcription of various cytokines, and in particu-

lar of interleukin (IL)-2, which is critical for T-cell proliferation and maturation. Unfortunately, chronic treatment with CsA is associated with CsA-specific side effects, e.g. hyperlipidemia, hypertension, nephrotoxicity, hirsutism and gingivall hypertrophy (2–6). The mechanisms by which CsA treatment induces these side effects are not completely understood but it is known that CsA increases mRNA levels for a number of genes involved in cholesterol biosynthesis and hypertension (2, 7). Hypertension can be the result of an abnormal sodium and water balance, endothelin-mediated systemic vasoconstriction and impaired vasodilatation secondary to reduction in nitric oxide (6, 7). CsA can, by inducing lipid metabolism disorders and hypertension, contribute to the development of allograft vasculopathy, a complication affecting many cardiac allograft recipients at the long term (1, 8). Nephrotoxicity is the third important side effect related to chronic CsA treatment. Two pathophysiologic mechanisms have been postulated by which CsA may exert its nephrotoxic effect. First, a vasomotor component of afferent arteriolar constriction reducing the glomerular perfusion, and second by replacement of vascular smooth muscle cells by hyaline material and the induction of interstitial fibrosis (9). Furthermore, hirsutism and gum hypertrophy are troublesome side effects commonly associated with the use of CsA.

Several studies have demonstrated that CsA stimulates cytokines, such as transforming growth factor (TGF)-β1 (10, 11). For instance, in CsA-treated animals with chronic nephropathy an association was found between the characteristic histological abnormalities in the kidney and increased renal expression of TGF-β1 (12, 13). Also hypertension and transplant coronary artery disease have been associated with elevated TGF-β1 levels (14, 15).

Primary treatment with tacrolimus (TAC), a more recently introduced calcineurin inhibitor that has a comparable mode of action to that of CsA, has been shown to be more effective in the treatment of ongoing rejection (16). Furthermore, both single- and multi-center trials have shown that treatment with TAC is accompanied with less *hyperlipidemia* and hypertension (17–19). In these studies it appeared that TAC also has nephrotoxic properties shown by renal dysfunction in TAC-treated cardiac allograft recipients. However, conversion studies from CsA to TAC in kidney allograft recipients showed that TAC treatment could significantly improve renal function (16, 20, 21).

The aim of this study was two-fold, first to assess if the conversion from CsA to TAC is associated both with immune activation and with improvement in CsA-related side effects in patients with cardiac transplantation and second, to analyze the involvement of growth factors and cytokines as underlying mechanisms related to the production of CsA toxic effects.

Material and methods

Patients

In 10 stable cardiac allograft recipients (9 males and 1 female, mean age 51.8 yr, range 16–60 yr; group I) time after transplantation 185 d (mean, range 60–450 d), we evaluated the effect of conversion from CsA to TAC. In these patients (group I) we studied the clinical parameters: blood pressure, renal function, and cholesterol level and the immunological parameters: intragraft IL-2 and IL-15 mRNA expression, and the TGF-β system. Forty-five cardiac allograft recipients (35 males and 10 females, mean age 48.8 yr, range 15–67 yr; group II) were converted from CsA to TAC because of hypertension (n = 27), hypercholestemia (n = 7), ongoing acute rejection (n = 4), gingival hyperplasia (n = 5) and general malaise (n = 2). Mean time after transplantation of these patients was 5.5 yr (range 0.5–14 yr). Maintenance immunosuppressive therapy consisted of CsA, prednisolone in all patients. In group II, 14 out of 45 patients also received the immunosuppressant mycophenolate mofetil.

All patients were on atorvastatin or pravastatin. Hypertension (systolic ≥ 150 mmHg and/or diastolic ≥ 100 mmHg) was treated by calcium-entry blockers (first choice) or angiotensis-converting enzyme (ACE) inhibition (second choice). In some patients, central working anti-hypertensive drugs had to be added. Comparable anti-hypertensive regimens were given to patients who were converted early or at a later time point after transplantation. Rejection was histologically diagnosed and graded according to the criteria of the International Society for Heart and Lung Transplantation (22). Anti-rejection therapy consisted of 1 g of methylprednisolone i.v. for 3 consecutive days. Frequently recurring or refractory rejection episodes were treated with rabbit-ATG.

Real-time reverse transcription (RT)-PCR

We used the real-time reverse transcription-polymerase chain reaction (RT-PCR) technique to quantify the amount of IL-2, IL-15, TGF-β1 and glyceraldehyde phosphate dehydrogenase

(GAPDH) mRNA expression in endomyocardial biopsy taken before (n = 15) and after conversion (n = 11) from patient group I. Messenger RNA extraction and cDNA transcription were performed as described in detail previously (23). The real-time reverse transcriptase polymenase chain reaction (RT-PCR) technique is based on the use of the 5′ nuclease activity of Taq DNA polymerase to cleave a nonextendable dual-labeled fluorogenic hybridization probe during the extension phase of PCR. The probe is labeled with a reporter fluorescent dye at the 5′ end and a quencher fluorescent dye at the 3′ end. Cleavage of the probe results in an increase in reporter flourescence at each cycle. Real-time PCR reactions are characterized by the point during cycling when amplification of the PCR product is first detected: the threshold cycle (C_t) that is defined as the fractional cycle number at which the flourescence generated by cleavage of the probe passes above baseline. The target message in endomyocard biopt (EMB) is quantified by measuring C_t and by using a standard curve using serial dilutions of a known amount of standard RNA. The choice of primer and probe (IL-2, IL-15) was defined using the primer express software (PE Biosystems, Norwalk, CT, USA). Nucleotide sequences for the oligonucleotide primers and probes are the following: IL-2 sense primer: 5′ TTT GAA TGG AAT TAA TAA TTA CAA GAA TCC 3′, IL-2 anti-sense primer: 5′ TTC TAG ACA CTG AAG CTG TTT CAG TTC, IL-2 probe 5′ CCA GGA TGC TCA CAT TTA AGT TTT ACA TGC CC 3′; IL-15 sense primer: 5′ TGG GTG AAT GTA ATA AGT GAT TTG AAA 3′, IL-15 anti-sense primer: 5′ AAC TCC AAG AGA AAG CAC TTC ATT G 3′, IL-15 probe 5′ TTT ATA TAC GGA AAG TGA TGT TCA CCC CAG TTG C 3′. Pre-developed Taqman® PDAR assays were used to measure TGF-β1 and the house keeping gene GAPDH concentrations (PE Biosystems). GAPDH was used in each sample to control sample-to-sample variations in mRNA concentrations.

Five microliters of cDNA was added to 45 μL of PCR mixture containing 25 μL of Universal PCR Master Mix (PE Biosystems), 1 μL of sense primer (50 pmol), 1 μL of anti-sense primer (50 pmol), 1 μL of FAM labeled probe (10 pmol), and 17 μL of H₂O. The PCR reaction was performed after a first step of 2 min at 50°C and 10 min at 95°C by 40 cycles of 15 s at 95°C and 1 min at 58°C (IL-2, IL-15) or 60°C (TGF-β1, GAPDH) using the ABI Prism 7700 sequence detector (PE Biosystems). All experiments were performed in duplicate. The relative concentration of intragraft IL-2, IL-15 and TGF-β1 was divided by the relative concentration of GAPDH. This represents the amount of target mRNA transcript corrected for the amount of mRNA used for RT and the efficacy of each reaction.

Flow cytometry

Whole blood ethylenediaminetetracetic acid (EDTA) samples were monitored for the presence of monocytes (CD14 positive) and lymphocytes (TCRαβ positive). Surface markers were analyzed by two-color cytometry using mAbs conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Quantitative flow cytometry was performed as described previously (24). Briefly, cells were stained with monoclonal antibodies directed to CD14 (Immunotech, Marseille, France), as the marker for monocytes, and WT31 (Immunotech) as the marker for TCRαβ positive cells, i.e. T-cells. In both monocytes and T-cells, TGF-β type I and type II receptors were monitored. For TGF-β type I receptor a two-step staining was used. After the first step with TGF-β type I receptor antibodies (30 min at 4°C), the cells were washed three times with 2 mL Hanks-Balanced Salt Solution (HBSS) (Gibco, Paisley, Scotland) supplemented with 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) and 0.1% sodium azide, followed by incubation with sheep-anti-rabbit IgG conjugated with PE for 30 min at 4°C Sanver Tech (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA). The antibody staining for TGF-β type II receptor was directly conjugated to PE Sanver Tech (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA).

The staining procedure was performed by incubating 100 μL whole blood with the above-mentioned monoclonal antibodies. After staining the red blood cells were lysed with FACS lysing solution (B&D) during 10 min. Samples were centrifugated and washed twice in cell Pack (TOA, Hamburg, Germany). Flow cytometric analysis was performed on a FACscan flow cytometer using Cell Quest software (B&D). From each tube 10 000 events in the gate were measured. To compare different flow cytometric assays in time we calibrated the flow cytometer using specific Calibration Beads (Calibration Beads Quantum 1000, Flow cytometer Standards Corp, San Jose, PR). Each bead contains a known amount of fluorochrome. The intensity of the flourescence is converted to a standard curve by the Quick Cal Program for Quantum Beads (B&D). The mean flourescence is denoted as molecular equivalents of soluble fluorochrome (MESF). Peripheral blood cells from healthy subjects (n = 12) served as controls (4 males, mean age 30 yr, range 24–52 yr).

Statistical analysis

Differences in distribution before and after conversion were assessed using the paired two-way variance test (paired Student's t-test). A level of $p \leq 0.05$ was considered to indicate statistical significance.

Results

Clinical data

We studied the effect of conversion from CsA to TAC in two cohorts of cardiac allograft recipients. Patients without overt CsA-related side effects (n = 10, group I) and those with CsA-related side effects (n = 45, group II). Clinical parameters studied in both cohorts included blood pressure, renal function and lipid levels.

Before conversion the mean whole blood trough CsA level for group I (no overt CsA-related side effects, mean time after transplantation 185 d) was 250 ± 65 and for group II (patients with CsA-related side effects, mean time after transplantation 5.5 yr) was 136 ± 57 ng/mL. After conversion the mean TAC trough level of these patients was 15.0 ± 3.8 ng/mL (group I) and 8.8 ± 2.7 ng/mL (group II). Concurrent medication was not changed.

Five out of 45 patients with overt CsA-related side effects (group II) were converted to TAC because of gum hypertrophy and 4 patients because of ongoing rejection episodes. The gum hypertrophy disappeared and the ongoing rejection resolved when patients received TAC. Conversion to TAC did not lead to histologically proven acute rejection episodes in any of the studied patients without signs of rejection at time of conversion.

Table 1 summarizes the results of the clinical parameters studied of the patients: blood pressure, renal function and lipid levels. For patient group I, converted to TAC within 2 yr after transplantation, and group II, patients who were switched to TAC because of CsA induced side effects, no positive effect of the conversion on renal function was found. The serum creatinine levels were comparable before and after conversion group I: 145 ± 43 vs 128 ± 28 μmol/L, p = 0.10, and group II 162 ± 56 vs 160 ± 68 μmol/L, respectively, p = 0.72. In contrast, a significant improvement on both blood pressure (systolic and diastolic) and total cholesterol levels was observed. Already at 8 wk after conversion, the systolic blood pressure of patient group I decreased from 154 ± 16 to 143 ± 21 mmHg and the diastolic blood pressure from 99 ± 11 to 90 ± 11 mmHg (p = 0.03 and p = 0.02, respectively), and for group II from 155 ± 17 to 142 ± 14 mmHg (systolic) and 99 ± 11 to 91 ± 8 mmHg (diastolic) (p < 0.001 and p < 0.001, respectively). Total cholesterol levels improved for patient group I from 6.6 ± 0.4 to 5.7 ± 0.3 mmol/L (p = 0.001), and for patient group II from 7.1 ± 1.7 to 6.1 ± 1.7 mmol/L (p < 0.001).

Cytokine and cytokine receptor expression before and after conversion

Intragraft mRNA expression of IL-2, IL-15 and TGF-β1 by real-time RT-PCR. We used the highly

Table 1. Effects of conversion from CsA to TAC on blood pressure, renal function and lipid levels

	Before conversion	After conversion	p value[1]
Patient group I (n = 10)[2]			
CsA tough level (ng/mL)	250 ± 65		
TAC trough level (ng/mL)		15.0 ± 3.8	
Blood pressure			
Systolic RR (mmHg)	154 ± 16	143 ± 21	0.03
Diastolic RR (mmHg)	99 ± 11	90 ± 11	0.02
Serum creatinine (μmol/L)	145 ± 43	128 ± 28	0.10
Total cholesterol (mmol/L)	6.6 ± 0.5	5.7 ± 0.3	0.001
Patient group II (n = 45)[3]			
CsA trough level (ng/mL)	136 ± 57		
TAC trough level (ng/mL)		8.8 ± 2.7	
Blood pressure			
Systolic RR (mmHg)	155 ± 17	142 ± 14	<0.001
Diastolic RR (mmHg)	99 ± 11	91 ± 8	<0.001
Serum creatinine (μmol/L)	162 ± 56	160 ± 68	0.72
Total cholesterol (mmol/L)	7.1 ± 1.7	6.1 ± 1.7	0.001

[1] Paired Student's t-test.

[2] Patients (group I, described in detail in the Material and methods) who were converted from CsA to TAC within the first 2 post-operative years.

[3] Patients (group II, described in detail in the Material and methods) who were converted from CsA to TAC because of CsA toxicity.

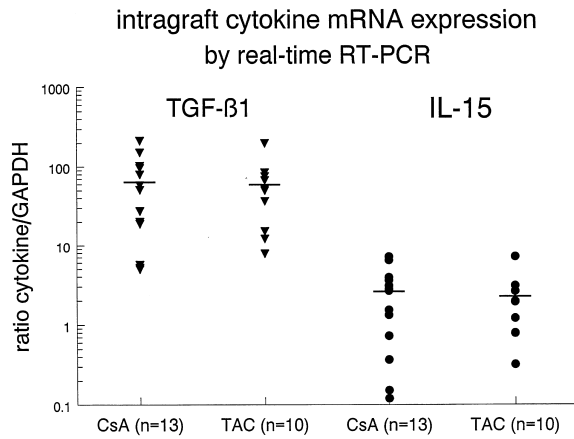## intragraft cytokine mRNA expression
by real-time RT-PCR

Fig. 1. Messenger RNA expression levels of TGF-β1 and IL-15 measured in endomyocardial biopsies real-time RT-PCR from patient group I before and after conversion from CsA to TAC. No significant differences were found in intragraft mRNA expression levels for TGF-β and IL-15 before and after conversion.

reproducible real-time RT-PCR to quantify intragraft IL-2, IL-15, and TGF-β1 mRNA expression in endomyocard biopt (EMB) from stable cardiac allograft recipients (n = 10, group I) before (n = 15) and after conversion at 8 wk (mean, n = 11) from CsA to TAC.

There was no difference in intragraft cytokine mRNA expression levels before and after conversion. Comparable intragraft TGF-β1 and IL-15 mRNA levels were measured (Fig. 1). The expression of IL-2, the cytokine associated with immune reactivity, was only detectable in 2/26 EMB. One specimen was positive during CsA treatment and one specimen during TAC treatment.

Peripheral expression of TGF-β1 type I and II receptors by quantitative flow cytometry. To assess the effects of immunosuppressive medication on the expression level of TGF-β1 receptors on peripheral T-lymphocytes (TCR αβ positive) and monocytes (CD14 positive) we used quantitative flow cytometry. The analysis was exclusively performed on peripheral blood samples from patient group I.

Only a few TCR αβ lymphocytes stained positive for the type I and type II TGF-β1 receptors, 2.1 ± 2.2% and 2.2 ± 2.7% (mean ± SD), respectively. The proportion of CD14 positive cells with TGF-β1 type I receptors was 44.6 ± 28.5% and with type II receptors 8.7 ± 7.2%. For TGF-β1 type I and type II receptors these percentages on TCR αβ positive and CD14 positive peripheral blood cells were not influenced by the conversion to TAC. However, conversion did affect the expression level of TGF-β1 type I receptor and not of TGF-β1 type

II receptor (Fig. 2). On both TCR αβ positive lymphocytes and CD14 positive cells the membrane bound expression of TGF-β1 type I receptor significantly decreased. Before conversion, during CsA treatment the expression of the TGF-β1 type I receptor on TCR αβ positive lymphocytes was MESF 9718 ± 4947 (mean ± SD) which decreased to MESF 6406 ± 2886 after conversion to TAC, and on CD14 positive monocytes from MESF 15263 ± 6979 to MESF 8012 ± 6181 (p = 0.02 and p = 0.002, respectively).

Discussion

In the present study, we have analyzed the consequences of conversion from CsA to TAC on both clinical and immune parameters of cardiac allograft recipients. Shortly after transplantation, within 2 yr (patient group I) the conversion to TAC resulted in improvement of blood pressure and cholesterol levels. This improvement in the studied clinical parameters was also seen in patients who were switched to the immunophilin TAC because of severe CsA toxicity. Even, as long as 14-yr post-transplant patients may benefit from conversion from CsA to TAC concerning hypertension, hypercholestemia and gum hyperplasia. Furthermore, the ongoing rejections resolved when patients were treated with TAC. These beneficial effects were accompanied with decreased expression of TGF-β type I receptor on peripheral mononuclear blood cells. Furthermore, a change in the immunosuppressive protocol did not lead to immune activation, neither histological signs of acute rejection nor increased IL-2 or IL-15 mRNA expression in EMB were seen.

Hyperlipidemia, hypertension and renal failure are very common following heart transplantation and the prevalence increases with time after transplantation (1). These complications are important non-immunologic risk factors in allograft recipients. High lipid levels may contribute to the onset and/or progression of chronic rejection, while renal insufficiency has a high impact on the mortality of the cardiac allograft recipients (1, 5). These and other complications are often related to chronic CsA treatment that can exert its adverse effects by disturbed cytokine production (10, 11). Therefore, the beneficial effects seen in our and other conversion studies might be the result of restored physiological levels of TGF-β1, a cytokine known for its role in CsA toxicity (12, 14, 17–19). Recently, we could indeed show that TGF-β1 plays a role in renal insufficiency in our CsA-treated cardiac allograft recipients as we found an association between TGF-β1 codon 10 genotypes and renal dysfunction
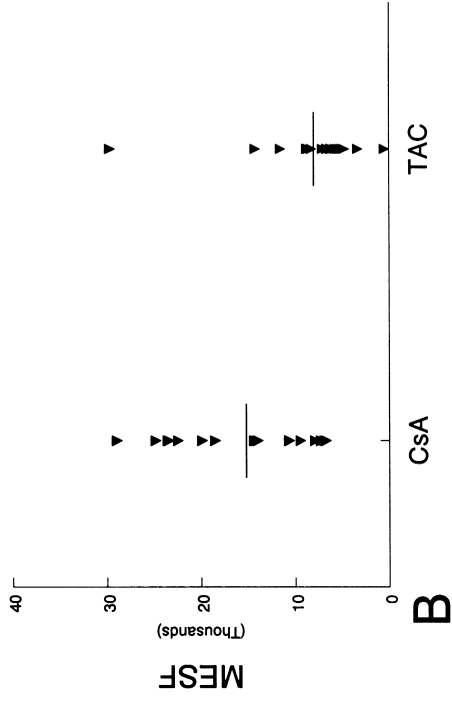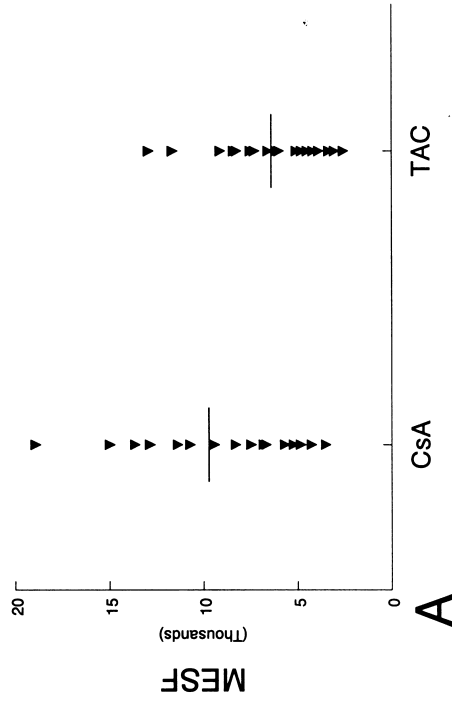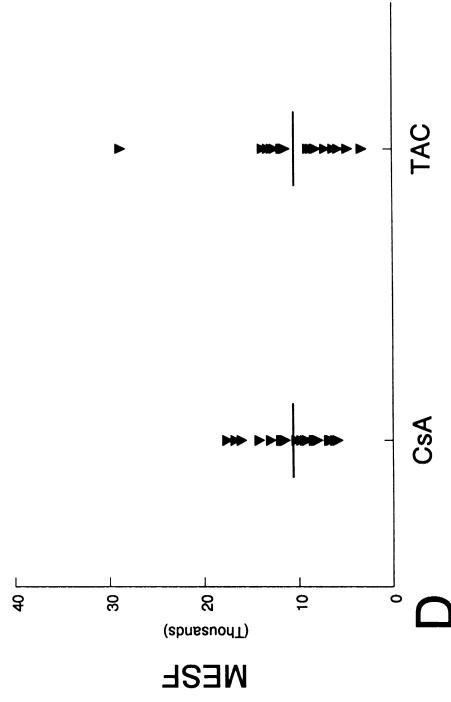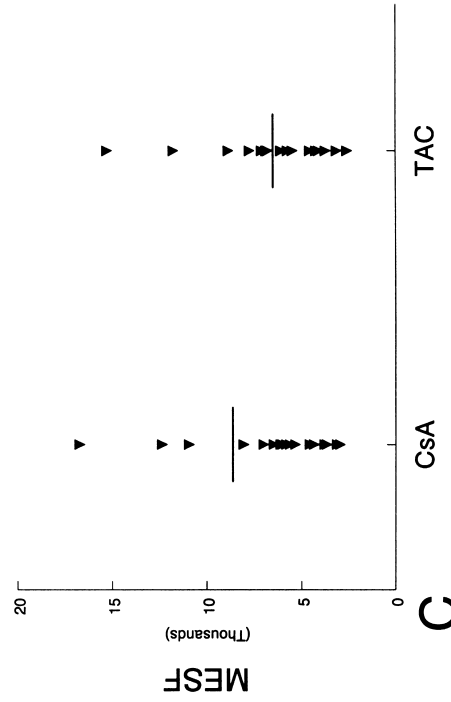
Fig. 2. By quantitative flow cytometry we determined the TGF-β1 type I and type II receptor expression levels on TCRαβ positive cells (T-cells) and on CD14 cells positive (monocytes). The mean flourescence is denoted as molecular equivalents of soluble fluorochrome (MESF). Conversion from CsA to TAC resulted in significantly lower TGF-β1 type I receptors on both T-cells and monocytes (A and B), p = 0.02 and p = 0.002, respectively. The expression of TGF-β1 type II receptors was not influenced by TAC treatment (C and D).

(serum creatinine $\geq$ 250 μmol/L) (25). In the present study, no effect of conversion was seen on the intragraft mRNA expression level of TGF-β (Fig. 1) as the activation of this particular cytokine is regulated at the protein level (26). Therefore, a possible relationship between TGF-β expression and CsA toxicity can be found by analyzing active TGF-β1 protein levels. In that prospect it is important to study both the TGF-β expression by parenchymal cells and graft infiltrating cells.

Cytokines act on target cells through high affinity membrane receptors. The active form of TGF-β1 binds to the TGF-β type I and type II receptors, which are signaling mediators (26). Nowadays, patients are often treated with the immunosuppressant TAC, a calcineurin inhibitor that competes with TGF-β type I receptor for FKBP12 interaction (26, 27). FKB12 is a binding protein for TAC (i.e. FK506). Thus, association of FKBP12 with TGF-β type I receptor decreases receptor signaling. In the present conversion study from CsA to TAC we confirmed that treatment with TAC reduced TGF-β type I receptor expression on peripheral blood cells. Also primary treatment with TAC is not associated with increased production of TGF-β1 (28, 29). These findings may explain why cardiac allograft recipients on a TAC-based immunosuppressive regimen have less hypertension and hyperlipidemia, and better renal function than patients treated with CsA (17–19).

In our patient population, conversion from CsA to TAC did resolve the ongoing rejections and resulted in significant improvement of systolic and diastolic blood pressure and lipid levels and the gum hypertrophy disappeared even after years of chronic CsA treatment. The beneficial effects of replacement of CsA by TAC on plasma cholesterol levels might be the result of shutting of the key gene involved in cholesterol biosynthesis, i.e. sterol regulatory element-binding protein (SREBP)-2 (2). However, despite improved blood pressure the conversion did not improve renal function reflecting either comparable intrarenal vasoconstriction based nephrotoxicity of both CsA and TAC leading to diminished glomerular filtration rate or structural kidney damage induced by long-term CsA therapy. Renal insufficiency induced by CsA is characterized by interstitial fibrosis, tubular atrophy and glomerular vascular lesions (30).

We conclude that conversion from CsA to TAC is of benefit for cardiac allograft recipients even for patients who were on CsA for years. The change in immunosuppressive regimen was associated with decreased TGF-β1 type I receptor expression on peripheral mononuclear blood cells, suggestive for the involvement of TGF-β1 in CsA–related complications.

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