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1α,25-Dihydroxyvitamin D_3 shows strong and additive immunomodulatory effects with cyclosporine A in rat renal allotransplants

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1α,25-Dihydroxyvitamin D_3 shows strong and additive immunomodulatory effects with cyclosporine A in rat renal allotransplants.

Background. Vitamin D_3 and its metabolites have long been found to exert immunosuppressive effects both in vivo and in vitro. The present study investigated the effect of 1α,25-dihydroxycholecalciferol (1,25DHC) on vascularized renal allografts in rats.

Methods. Three days prior to transplantation, two groups of animals were subjected to 1,25DHC (1 μg/kg/day IP) and a low calcium diet, which was continued until the end of the experiments. Recipient organs were removed and single allografts were transplanted in a high responder strain combination (ACI → Lewis). Following transplantation, low-dose cyclosporine A (3.2 mg/kg/day CsA) administration was started in two experimental groups of recipients (one group receiving 1,25 DHC additionally) whereas the control allograft recipients received no immunosuppression (control III). Graft survival and renal function was monitored until death or the end of experiments and allograft rejection was assessed histologically using the Banff classification.

Results. 1,25DHC significantly prolonged allograft survival in comparison to control III (9.6 ± 1 vs. 5.7 ± 0.2 days; $P = 0.009$). In addition, a combination of 1,25DHC and low-dose CsA increased allograft survival compared to CsA administration alone (24 ± 0.9 vs. 13 ± 0.3 days; $P = 0.008$). 1,25DHC preserved renal creatinine clearance and decreased proteinuria in comparison to control III, and the combination of 1,25DHC and low-dose CsA again showed an additive effect on preservation of renal function. 1,25DHC and low-dose CsA both decreased interleukin (IL)-2 and IL-12 expression levels in serum and allografts, and a combination treatment produced the strongest attenuation of IL-2 and IL-12 expression. In addition, 1,25DHC increased IL-4 and IL-10 expression levels in allografts, whereas CsA alone did not alter IL-4 and IL-10 expression. In contrast, combination of 1,25DHC and low-dose CsA showed a significant increase in IL-10 expression levels whereas IL-4 expression was not elevated.

Conclusion. Monotherapy with 1,25DHC significantly prolongs survival of renal allografts and preserves graft function in rats. A combination of 1,25DHC and CsA caused an additive effect on graft survival with differential regulation of pro- and anti-inflammatory cytokines, as compared to 1,25DHC administration alone.

Key words: immunosuppression, graft survival, cytokine regulation, transplantation, cell cycle, vitamin D.

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Throughout the 1990s, transplantation has seen a surge of synthetic and naturally occurring compounds with immunosuppressive properties. The rationale for this search is the development of an optimal drug regimen with highly selective immunosuppressive properties and minimal side effects. Among those compounds, sterols like corticosteroids and the hormonally active vitamin D metabolite, 1α,25 dihydroxy cholecalciferol (1,25DHC), are physiologically occurring molecules with immunomodulatory effects in humans [1]. To date, vitamin D has not been not used in clinical practice because of its hypercalcemic effects. Nevertheless, the immunomodulatory effects of 1,25DHC have been studied intensively in lectin- or antigen-activated lymphocytes in vitro as well as in autoimmune disease models [2, 3]. Thus, 1,25DHC inhibits T helper subset (Th1) functions and attenuates the secretion of interleukin 2 (IL-2) and interferon γ (IFN-γ) [4]. Furthermore, 1,25DHC specifically inhibits in a time and dose-dependent fashion the generation of cytotoxic natural killer cells and this effect can be attenuated by administration of exogenous IL-2 [5]. Similarly, Meehan, Kerman and Lemiere have shown that 1,25DHC prevents the generation of cytotoxic T cells and promotes T-suppressor cell activity in an in vitro model of allograft response [6]. Moreover, 1,25DHC induces an arrest in the G1 phase of the T-cell cycle and induces apoptosis in stimulated T lymphocytes whereas exogenous IL-2 reverted both the cell cycle block and apoptosis [7].

In addition, it was recently reported that 1,25DHC reduces IL-12 expression in macrophages and dendritic cells [8, 9]. These results indicate that 1,25DHC may

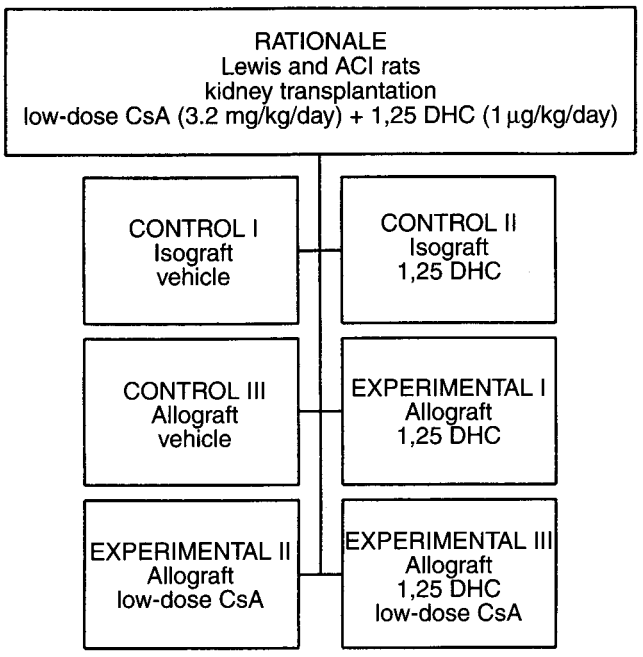

Fig. 1. Flow chart outlining the different control and experimental groups.

exert beneficial effects in situations where a Th1 T-cell differentiation arises, and thus may improve outcome in allogenic transplant models. Other data suggest that derivatives of vitamin D also may potentiate the effect of calcineurin inhibition in vitro and in vivo [10–12]. We therefore investigated the immunosuppressive properties of 1,25DHC as a sole anti-rejection treatment as well as in combination with a calcineurin inhibitor in an arterialized rat renal transplant model. Following transplantation, graft function and survival were assessed and expression levels of pro- (IL-2, IL-12) and anti-inflammatory cytokines (IL-4, IL-10) were determined. Furthermore, allograft rejection was estimated histologically and classified according to the Banff 97 criteria [13]. In addition, osteopontin expression levels were assessed in allografts in order to investigate whether vitamin D exerts all immunomodulatory effects at a set of traditional inflammatory foci or whether vitamin D also modulates alternate events in the course of allograft lifespan.

METHODS

Animals and experimental design

Animal experiments were performed in accordance with the national guidelines for the care and use of laboratory animals and were approved by the local authorities (Fig. 1). Male inbred Lewis and ACI rats (250 to 320 g), obtained from Harlan Sprague-Dawley (Horst, The Netherlands), were used in a high responder strain combination (ACI → Lewis). All animals were placed on an experimental diet containing 0.47% calcium, seven days prior to and following transplantation [14]. Tap water was given ad libitum. Recipient animals (male Lewis rats) were divided into three control and three experimental groups (N = 6 to 9/group). Control I were subjected to isograft transplantation (Lewis → Lewis) and vehicle administration only. Control II consisted of isograft recipients receiving daily intraperitoneal injections of 1,25DHC, starting three days prior to and following transplantation until harvesting of organs. Control III underwent allotransplantation (ACI → Lewis) and received daily intraperitoneal vehicle injections only. Experimental animals (EXP) I received allotransplantation and daily intraperitoneal injections of 1,25DHC. EXP II animals were provided with allotransplantation and low dose cyclosporine A (CsA) administration, whereas EXP III animals were subjected to allotransplantation and a combination of intraperitoneal low-dose CsA and 1,25DHC injections.

Immunosuppressive therapy

Vitamin D_3. The bioactive compound 1,25DHC (Rocaltrol®; Roche Pharma, Basel Switzerland) was solubilized in ethanol and diluted in saline before being injected into recipients. Administration in allograft recipients was started three days prior to and following transplantation until harvesting of organs. A daily dosage of 1 μg/kg was found to be effective in previous studies with rat cardiac allografts [14], and was chosen for all animals receiving 1,25DHC. Based on our previous experiments with a rat animal model, intraperitoneal administration of 1,25DHC was preferred over oral vitamin D_3 intake for two main reasons. First, oral food consumption was inconsistent during the first three to four days following transplantation [15]. Second, animals were housed in pairs (two animals per cage) in the central animal facility. Thus, oral administration of 1,25DHC by incorporation into the daily diet would have resulted in an unpredictable dosage per animal.

Cyclosporine A. Cyclosporine A (Sandimmune® injection grade) was obtained from Novartis Pharma (Basel, Switzerland) and diluted in saline. CsA was given IP at a dosage of 3.2 mg/kg/day starting perioperatively and continuing until harvest of organs. The dose of CsA was chosen based on the reports of others [16].

Kidney transplantation experiments

In order to create a life-sustaining model, a single kidney transplant model with bilateral recipient nephrectomy was used. For transplantation, recipients were anesthetized with pentobarbitone sodium IP (50 mg/kg body weight). After a median laparotomy, both kidneys were removed and single allograft kidney transplantation with the ACI (Rt.1.a) to LEWIS (RT.1.l) strain combination (Control III, EXPs I to III) or single syngeneic transplants (Controls I and II) were performed. Donor kidneys were rinsed

with 5 mL Ringer's lactate solution and transplanted orthotopically. Renal vessels were anatomized using a 10/0 Prolene® suture and ureters were connected via intraluminal polyethylene cuffs. Transplantation time ranged from 15 to 20 minutes and was not different between groups.

After transplantation, all animals were monitored and kept in metabolic units for renal function analysis. Urine volume, urine protein and urea levels as well as serum creatinine concentration and creatinine clearance were measured every second day. In addition, to study the effect of 1,25DHC on calcium metabolism, serum calcium levels were measured with a Hitachi multi-analyzer (Hitachi, Zurich, Switzerland) on day −3, 0, 5, 9, 14 and 21.

In separate sets of experiments (N = 6/group), renal allografts were taken for histology and cytokine determination on day 0, 5, 9, 14 and 21 post-transplant. Histological changes were graded according to the Banff 97 working classification of renal allograft pathology, in a blinded fashion [13].

Determination of cytokines in serum and tissue homogenates (IL-2, IL-4, IL-10 and IL-12) by ELISA

Graft tissue and peripheral blood samples were harvested from transplanted animals at defined time points and subjected for ELISA assays for determination of individual IL's as described by the manufacturer (PharMingen, San Diego, CA, USA). Homogenates from contralateral donor kidneys as well as serum samples from recipient animals prior to grafting were assessed to determine baseline IL concentrations. Briefly, plates were coated overnight with the capture antibody at 4°C in 0.1 mol/L carbonate buffer, pH 9.5 (1 μg/mL for anti-IL-2, -4 and -10, PharMingen; and 1 g/mL for anti-IL-12, Biosource, Camarillo, CA, USA). The coated plates were washed six times with 0.01 mol/L, pH 7.4 phosphate buffered saline with 0.05% Tween-20 (PBS-T). Samples were incubated for two hours at 37°C, washed six times with PBS-T, and incubated for an additional two hours with the biotylinated secondary antibody [biotylinated monoclonal anti-rat antibody for IL-2, IL-4 and IL-10 (PharMingen), in PBS-T and biotylinated polyclonal anti-rabbit antibody for rat IL-12 (Biosource USA)]. The plates were washed six times and incubated for one hour at room temperature with avidin-peroxidase (Sigma, St. Louis, MO, USA) 1:400 in PBS-T. The plates were washed and developed with ortho-phenylamine-diamine (Sigma) in 1% methanol with 0.003% hydrogen peroxide. The reaction was stopped by the addition of 50 μL of 8 N sulfuric acid and optical density (OD) was determined at 492 nm. All assays were performed in triplicate for determination of interassay variability. A five point standard curve was run in triplicate with each assay. Optical density values at each test point were corrected by subtracting OD readings obtained for carbonate buffer only.

Determination of renal osteopontin expression by Western blot analysis

Total protein was prepared by homogenizing graft kidney tissue in 25 mmol/L Tris (pH 7.5), 50 mmol/L NaCl, and 10 mmol/L ethylenediaminetetraacetic acid (EDTA) containing protease inhibitors (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). Aliquots containing 40 μg of protein were subjected to sodium dodecyl sulfate (SDS)/12% polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Membranes were incubated with a monoclonal anti-osteopontin antibody (Developmental Studies Hybridoma Bank, University of Iowa, Aimes, IA, USA) diluted 1:1000, washed and subjected to chemiluminescence Western blotting using a commercially available blotting kit (Pierce Chemical Co., Rockford, IL, USA) and a horseradish peroxidase-conjugated goat anti-rabbit antibody (Developmental Studies Hybridoma Bank). The intensity of the Western blot signal was quantified with a CCD camera (Fuji film LAS-1000; Raytest, Urdorf, Switzerland) using the software AIDA 2.1 (Raytest, Urdorf, Switzerland) and normalized to β-tubulin (H235) protein expression levels (rabbit polyclonal IgG, 200 μg/mL; Santa Cruz Biotechnology, Heidelberg, Germany) in order to equalize differences in protein loading.

Statistics

Results are expressed as mean values ± SD. Graft survival was calculated with the Kaplan-Meier product limit estimator. Differences in survival rates between the various groups were tested with the log rank test in a statistical package program (SPSS® Statistical Software, Chicago, IL, USA). For enzyme-linked immunosorbent assay (ELISA), Western blot assays and kidney function assessment differences between groups were analyzed by analysis of variance (ANOVA) with the Bonferroni correction. A probability of $P < 0.05$ was considered statistically significant.

RESULTS

Effect of 1,25DHC on survival

Kidney isograft transplantation and vehicle administration (control I, N = 6) did not lead to recipient death or major graft dysfunction (Fig. 2). Similarly, isograft transplantation under 1,25DHC medication was well tolerated and the graft recipients remained alive (control II, N = 6). In contrast, in a high responder strain combination (ACI → LEW), vehicle administration (control III; N = 9) led to a mean survival of 5.7 ± 0.2 days (range 5 to 6 days). 1,25DHC (EXP I) significantly increased survival when compared to control III (mean 9.6 ± 1.2 days, $P = 0.009$). Low-dose CsA treatment (EXP II, $N = 9$) prolonged survival in comparison to EXP I (mean 13 ±

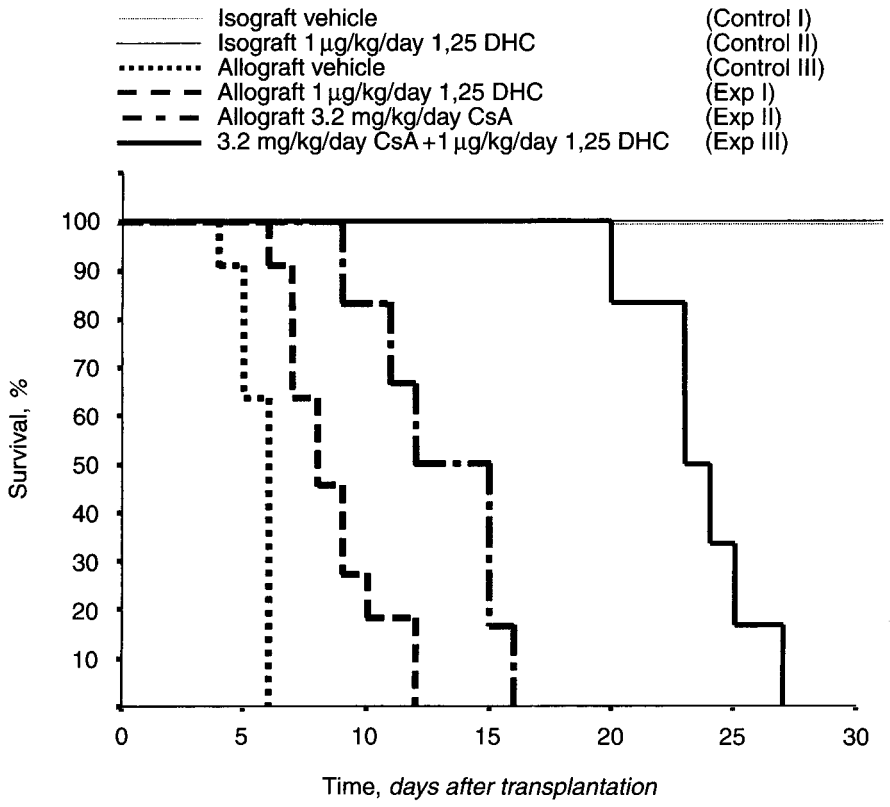

Fig. 2. 1,25-Dihydroxycholecalciferol (1,25DHC) improved survival following renal allograft transplantation Kaplan-Meier survival curves of rat recipients of an orthotopic vascularized renal isograft/allograft. Allografts were performed in the high-responder ACI → Lewis rat strains combination. Animals treated with 1,25DHC survived longer than animals receiving vehicle only. In the combination regimen there was a strong additive effect.

0.3 days, range 11 to 15 days; $P = 0.008$). The combination of 1,25DHC with low-dose CsA (EXP III, $N = 9$) further increased survival (mean 24 ± 0.9 days, range 20 to 27 days) when compared to low-dose CsA only ($P < 0.001$).

Effect of 1,25DHC on graft function

Isograft recipients (Control I) showed an unaltered graft function throughout the experiments as shown by the creatinine clearance and urine protein excretion values (Fig. 3). Administration of 1,25DHC did not influence isograft function at all (Control II; data not shown). Allograft transplantation without administration of immunosuppressive agents (Control III) led to a rapid deterioration of graft function. 1,25DHC treatment decelerated the deterioration of creatinine clearance and prevented proteinuria significantly during the first seven days after transplantation when compared with normal allograft rejection. Low-dose CsA administration conserved creatinine clearance and prevented proteinuria until day 9 following allografting. Combined administration of low-dose CsA and 1,25DHC preserved renal function until the third week following allograft transplantation.

Effect of 1,25DHC on IL-2, IL-4, IL-10 and IL-12 production

Prior to transplantation (day 0), low levels of IL-2, IL-10 and IL-12 concentrations were detectable in most of the animals, whereas IL-4 was not detectable in the plasma or in the intragraft homogenates (Fig. 4 A–G). Pretreatment with 1,25DHC did not alter the cytokine expression pattern prior to transplantation. Similarly, 1,25DHC administration (Control II) showed no effect on the various cytokine expression patterns during the experimental period in comparison to vehicle treated isograft recipients (Control I). On the fifth day following allograft transplantation, IL-2 levels rapidly increased in vehicle treated animals (Control III) both in serum and kidney grafts, whereas the 1,25DHC treatment (EXP I) prevented an increase of IL-2 levels until day 9 following the allograft transplantation. Administration of CsA alone or in combination with 1,25DHC did not increase IL-2 levels both on day 5 and day 9 (EXP II and III) compared to pre-transplantation values. However, the combined administration of CsA and 1,25DHC caused a significantly smaller increase in serum IL-2 levels on day 14 compared to CsA treatment alone ($P < 0.002$, Fig. 4A).

Similar results were observed for IL-12 expression. Baseline concentrations were identical at time of transplantation (day 0) for all groups of animals. Allograft transplantation caused a significant increase in either serum ($P < 0.001$) or kidney graft expression ($P < 0.001$) at day 5. Intragraft concentration of IL-12 was tenfold increased when compared to peripheral levels. Administration of 1,25DHC significantly inhibited IL-12 expression both in serum and allografts at day 5 ($P < 0.001$) and day 9

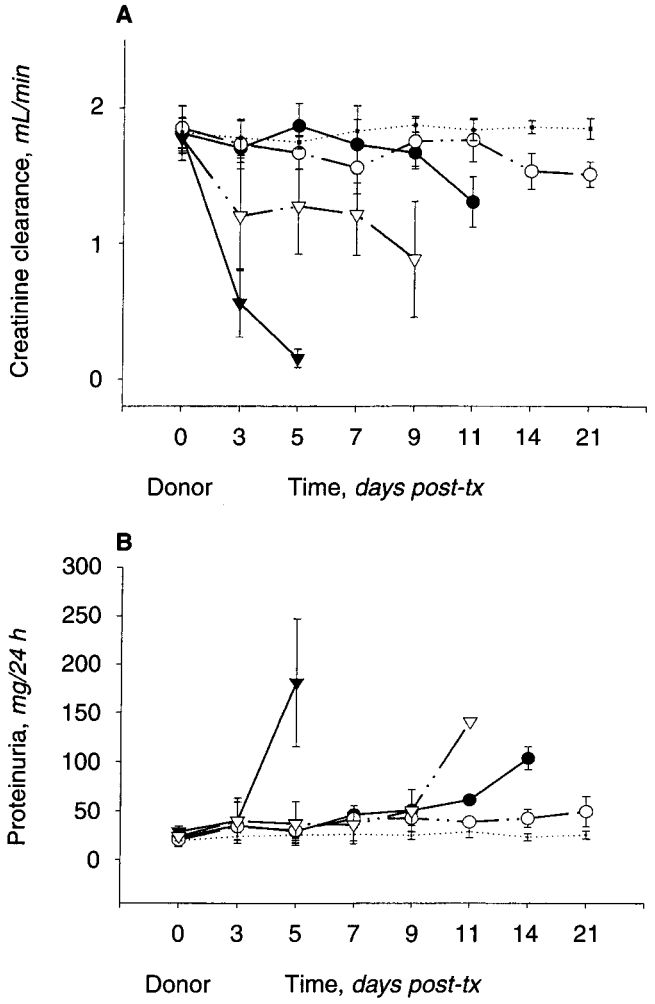

Fig. 3. 1,25DHC treatment improved the renal function of rats that were recipients of a renal allograft, according to serum creatinine clearance (*A*) and proteinuria (*B*). Animals treated with 1,25DHC showed significantly improved renal function in a mono- and combination therapy model. Values obtained in animal recipient of an isograft (Control I+II) are shown in open triangles. The data represent the mean ± SD of six animals per group. Symbols are: (■) isograft vehicle, Control I group; (▼) allograft vehicle, Control III group; (▽) allograft 1,25DHC, Exp I; (●) allograft CsA, Exp II; (○) allograft CsA + 1,24DHC, Exp III.

($P < 0.001$; Fig. 4 C, D) when compared to vehicle treated animals (Control III). A more pronounced attenuation of IL-12 expression was achieved with low-dose CsA administration and the most significant effect was observed with a combination of low-dose CsA and 1,25DHC treatment.

Interleukin-10 serum levels increased slightly five days following allotransplantation (Control III) whereas intragraft expression levels remained unaltered. In contrast, administration of 1,25DHC caused a significant increase in IL-10 expression levels both in serum and allografts at day 5 compared to Control III ($P < 0.001$) and a further increase in IL-10 expression levels was noted at day 9. In comparison to controls, a lesser but still significant increase in IL-10 concentrations were noted in animals receiving a combination of 1,25DHC and CsA following allograft transplantation, whereas administration of CsA alone did not affect IL-10 expression levels during the study period (Fig. 4 E, F).

Intragraft expression levels of IL-4 were unaltered following transplantation with the exception of 1,25DHC receiving allografts (EXP I), which showed a marked increase five and nine days following transplantation (Fig. 4G). Serum IL-4 expression levels were not detectable in any animals at all time points.

Effect of 1,25DHC on renal osteopontin expression

Osteopontin (OPN) protein levels on day 5 following isografted transplantation (Control I and II) did not alter (Fig. 5). In contrast, allografting caused a substantial upregulation of OPN protein expression in vehicle treated animals (Control III) at day 5 whereas administration of 1,25DHC (EXP I) decreased OPN expression levels significantly ($P = 0.004$). CsA treatment (EXP II) inhibited renal OPN expression until day 9, while the combined therapy with CsA and 1,25DHC (EXP III) led to a reduction in OPN protein expression until day 14 following allografting (EXP III vs. EXP II, $P < 0.001$).

Histological assessment of graft rejection

The Banff 97 grading for each group is shown in Table 1. Control III ($N = 6$) showed histological changes compatible with severe acute rejection at day 5 such as marked necrosis in the cortex, moderate-to-severe tubulointerstitial mononuclear infiltrates and moderate-to-severe intimal arteritis. In addition, two cases showed transmural arteritis with fibrinoid changes and medial smooth muscle necrosis (Fig. 6A). Furthermore, several areas of tubular basement membrane destruction were found. In the vitamin D_3-treated group (EXP I), the histological alterations were less severe revealing trivial tubulointerstitial inflammation and no signs of arteritis could be detected (Fig. 6B). Administration of CsA (EXP II and III) lead to mild tubulointerstitial mononuclear infiltration at day 14 following allotransplantation. All allografts of animals subjected to low-dose CsA only showed severe intimal arteritis compromising <25% of the luminal area (Banff type IIB) or transmural arteritis with necrosis of medial smooth muscle cells (Banff type III) at day 14. In contrast, none of the allografts of the CsA/1,25DHC group were graded higher as Banff IIA.

Toxicity

A significant limitation to the use of vitamin D_3 derivatives in transplantation is the development of hypocalcemia. Significant hypocalcemia was not observed in 1,25DHC treated recipients. Serum calcium levels in recipients treated with 1,25DHC (Control II, Exp I and III) increased slightly but non-significantly increased during the study period in comparison to animals not subjected to 1,25DHC (Table 2). Low-dose CsA administration achieved mean serum CsA levels of 245 ± 34 ng/mL.

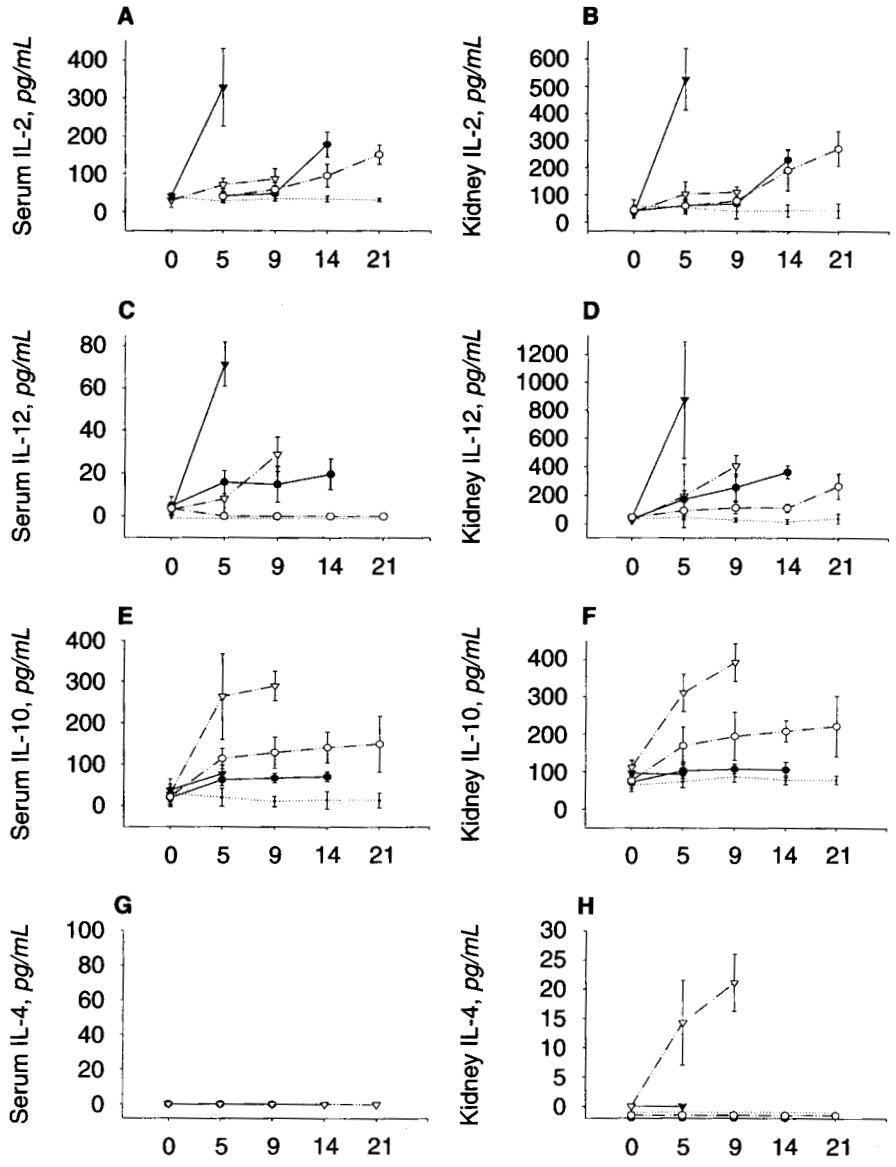

Fig. 4. Serum and intragraft cytokine expression levels. Evaluation of serum and kidney interleukins at baseline and during the transplant course. Expression levels of IL-2 (*A*, serum and *B*, intragraft) were markedly decreased in animals receiving 1,25DHC and/or low-dose CsA. Similar results were observed for IL-12 expression (*C*, serum and *D* intragraft). In contrast, IL-10 expression was significantly increased under 1,25DHC administration whereas low-dose CsA did not alter IL-10 expression levels (*E*, serum and *F*, intragraft). Serum IL-4 was not detectable but 1,25DHC caused a strong induction of intragraft IL-4 expression following allografting in comparison to vehicle treated animals (*G*). In contrast, low-dose CsA administration did not alter IL-4 expression neither as single agent treatment nor in combination with 1,25DHC (*G*). The data represent the mean ± SD of six animals per group. Symbols are: (■) isograft vehicle, Control I group; (▼) allograft vehicle, Control III group; (▽) allograft 1,25DHC, Exp I; (●) allograft CsA, Exp II; (○) allograft CsA + 1,24DHC, Exp III.

Table 1. Histological grading of acute allograft rejection according to the Banff 97 classification

Group	Drug	*N*	Acute rejection type (n)*		
			Day 5 post-Tx	day 9 post-Tx	Day 14 post-Tx
Control III (allograft)	Vehicle	6	IIA, IIB (3), III (2)		
EXP I	1,25 DHC 1000 ng/kg	6	normal, borderline (5)	IIA (2), IIB (2)	
EXP II	CsA 3.2 mg/kg	6	normal (2), borderline (4)	borderline (2), IA (4)	IIB (3), III (3)
EXP III	CsA 3.2 mg/kg + 1,25DHC 1000 ng/kg	6	normal, borderline (5)	borderline (3), IA (3)	IA, IB (2), IIA (3)

*Numbers in parentheses indicate the numbers of individuals graded for that type. Groups are defined in the text.

DISCUSSION

The remarkable biological effects of vitamin D analogs make them prime immunosuppressive agents in a transplant setting. For instance MC1288, a vitamin D analog, prolonged cardiac and small bowel allograft survival in rats and furthermore prevented histological manifestations of chronic rejection by inhibiting the inflammatory response of aortic allografts [17]. Previously, we have demonstrated that 1,25DHC inhibited neonatal as well as vascularized heart transplant rejections more effectively than a high-dose CsA regimen [14]. Moreover, 1,25DHC treated mice were less susceptible to infections and

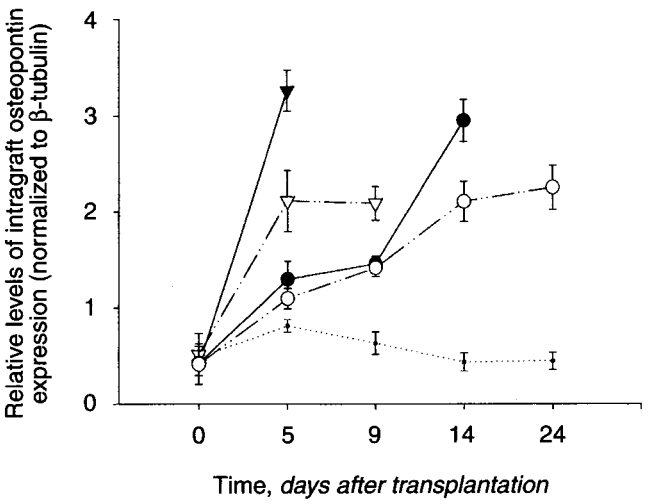

Fig. 5. Intragraft osteopontin protein expression Western blot analysis comparing the effects of vehicle, single and combined administration of 1,25DHC and low-dose CsA on renal osteopontin (OPN) expression. Results are representative of six independent probes/group/day. β-tubulin structural protein was used as internal control. Relative osteopontin expression levels were normalized to β-tubulin values and shown as OPN/β-tubulin ratio. 1,25DHC caused a significant reduction of osteopontin protein expression in renal allografts when administered either as single agent or in combination with low-dose CsA. Symbols are: (■) isograft vehicle, Control I group; (▼) allograft vehicle, Control III group; (▽) allograft 1,25DHC, Exp I; (●) allograft CsA, Exp II; (○) allograft CsA + 1,24DHC, Exp III.

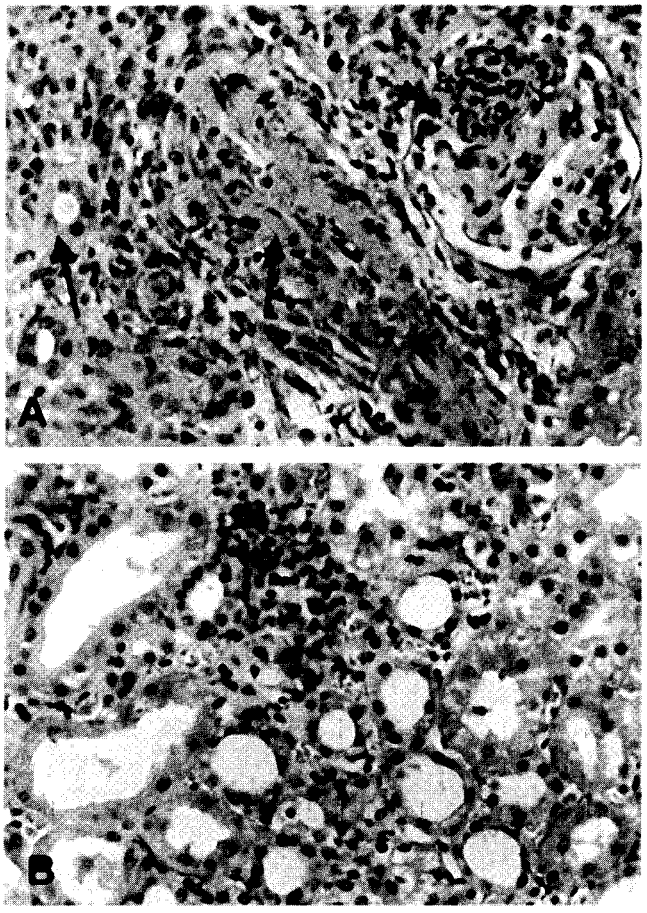

Fig. 6. (*A*) Control allograft with transmural arteritis and fibrinoid necrosis of the vessel wall. Note the severe interstitial mononuclear infiltrate and the presence of tubulitis (arrows). One glomerulus is infiltrated by neutrophil granulocytes (dotted arrow). (*B*) Vitamin D_3-treated allograft showing mild interstitial mononuclear infiltrates and questionable tubulitis (H&E, magnification ×400).

showed less bone loss than CsA treated animals [18]. The data presented here demonstrate that administration of 1,25DHC significantly prolonged renal graft survival in a high responder strain combination in rats. Thus, our results are in agreement with previous reports examining the effects of vitamin D_3 analogs on heart, aortic and small bowel allografts [17–19]. In contrast, Lemire et al could not observe a difference in graft survival using a neonatal non-vascularized murine heart transplantation model [20]. Similarly, Jordan et al reported a minimal effect of 1,25DHC on skin graft survival and rat cardiac allograft survival [21]. Both studies found significant toxicity. One reason for the controversial findings may be based on differences in experimental setup, which limited the possibility to compare these data. In addition, there are considerable differences in the route of 1,25DHC administration and daily dosage used in these different studies. Furthermore, several studies used various analogs of 1,25DHC with fewer calcemic effects, and therefore higher doses were administered in some of these studies, which may explain the more pronounced effect on graft survival [18, 20, 21].

In clinical transplantation, a high-dose vitamin D_3 treatment under close monitoring of urine and serum calcium levels might be used as an induction therapy to reverse the initial acute rejection episode and to conserve graft function. Alternatively, the immunosuppressive effect of 1,25DHC could be used in combination with other immunosuppressants in order to potentiate the anti-rejection therapy but minimize side effects. Additive effects of CsA in combination with derivatives of 1,25DHC have been reported in cardiac transplantation [12] as well as in syngeneic islet grafts in spontaneously diabetic non-obese mice [11] and in skin allografts in mice [22]. Our results on rat renal allograft survival in animals subjected to a combination therapy of low-dose CsA and 1,25DHC revealed a superior survival compared to only single agent therapy. Thus, our data correspond well with previous reports and may confirm that vitamin D analogs are potent additive drugs for other immunomodulators [11, 12, 22]. This is particularly relevant since many patients are taking vitamin D supplementation in order to prevent bone loss following kidney transplantation.

An important additional finding of our experiments is that 1,25DHC markedly attenuated renal allograft rejection. Grafts in animals receiving vitamin D_3 showed

Table 2. Calcium assessment

Group	Drug	*N*	Serum calcium levels *mg/L ± SD*				
			Pre-study Day −3	Day 0 post-Tx	Day 5 post-Tx	Day 9 post-Tx	Day 14 post-Tx
Isograft control I	Vehicle	6	93 ± 6.3	93 ± 7.6	86 ± 5.1	95 ± 9.5	94 ± 8.9
Isograft control II	1,25DHC 1000 ng/kg	6	94 ± 8.9	98 ± 4.1	99 ± 5.3	102 ± 8.9	103 ± 5.2
Allograft control III	Vehicle	12	92 ± 2.9	91 ± 4.4	87 ± 10.2 (6)		
EXP I	1,25DHC 1000 ng/kg	12	95 ± 6.4	97 ± 4.9	105 ± 9.1 (10)	103 ± 6.3 (4)	
EXP II	CsA 3.2 mg/kg	6	91 ± 5.9	94 ± 2.3	89 ± 7.4	92 ± 5.3	93 ± 5.1
EXP III	CsA 3.2 mg/kg + 1,25DHC 1000 ng/kg	6	94 ± 3.3	98 ± 6.1	99 ± 4.1	104 ± 9.3	105 ± 7.3

Numbers in parentheses indicate the numbers of individual survivors determined. Serum calcium assessment in rats that received renal syngeneic and allograft 1,25DHC did not significantly alter serum calcium levels while under a low calcium diet during the early and late phases of the transplant course.

significantly less severe intimal arteritis whereas control allografts were graded as severe stages of acute rejection. Moreover, vitamin D_3 treatment was associated with a significant decrease in osteopontin expression following allografting. In the past, there has been considerable concern that active vitamin D might have a direct impact on tubulointerstitial and glomerular integrity by affecting the responses to tubular injury [23]. In our study, long-term vitamin D_3 administration to isograft recipients did not impair renal function in isograft recipients even after three weeks of continuous administration. In addition, there was no tubular or glomerular cell injury detectable histologically and osteopontin expression was not altered. Similarly, Schwarz et al documented that 1,25DHC is able to reduce progression of glomerulosclerosis and albuminuria in subtotal nephrectomized rats [24]. Furthermore, administration of various vitamin D analogs in mice for over one year did not cause kidney damage as revealed by extensive macroscopic and microscopic autopsy studies [25].

In our study, 1,25DHC significantly improved allograft function as indicated by the superior creatinine clearance and decreased urine protein excretion early and late in the transplant course. In the present study we administered intraperitoneal 1,25DHC to prevent inconsistent drug intake between individual animals during the first days post-transplant, as we have preliminarily observed in our experiments. Therefore, the administration route and the dose were based on previous experience [15]. Although an orally given vitamin D regimen comes closer to the clinical practice of vitamin D supplementation, we used the intraperitoneal regimen in all control and experimental groups to ensure compatibility between individual groups during the study period.

Consistent with previous findings from in vitro and in vivo studies, 1,25DHC caused a marked inhibition of both IL-2 and IL-12 cytokine expression in renal allograft recipients [8, 25–28]. At the cellular and molecular level, vitamin D not only targets helper T-cell activity by inhibiting the secretion of IL-2 and by suppressing the secretion of pro-Th1 cytokine IL-12 by antigen presenting cells, it also affects maturing dendritic cells, leading to enhanced IL-10 secretion upon activation by CD40 ligation [9]. Our study also detected an increase of IL-10 expression levels in all rats medicated with 1,25DHC. Several recent studies have shown that IL-10 plays a pivotal role in allograft rejection and that increased IL-10 expression levels may enhance allograft survival [29–33]. However, it has been shown that 1,25DHC increases IL-4 protein and transcription levels and that the immunoregulatory effects of 1,25DHC are much less effective in IL-4-deficient mice, suggesting that IL-4 production is a significant mediator in the action of vitamin D_3 on the immune system [34]. Therefore, we have determined the differential expression of IL-4 and IL-10 in our experimental groups and could observe that allografts in recipients subjected to 1,25DHC showed significantly increased levels of IL-4. Thus, our results provide further evidence that 1,25DHC exerts immunomodulatory effects by shifting the immune response from the Th-1 pathway to the Th-2 response, and that Th-2 related cytokines such as IL-4 and IL-10 may be involved in graft tolerance.

In a subsequent set of experiments, we examined the effects of a combined administration of low-dose CsA and 1,25DHC on cytokine expression, since 1,25DHC seems to interfere at a downstream level of calcineurin in the T-cell activation cascade [10]. Combination therapy of CsA and 1,25DHC resulted in the strongest Th-1 cytokine inhibition. In addition, IL-10 expression levels significantly increased whereas IL-4 expression did not alter in comparison to single CsA administration. The explanations for this observation remain speculative. It may be hypothesized that a combination of 1,25DHC and CsA has an additive effect on Th-1 cytokine inhibition. Alternatively, 1,25DHC could influence alternate pathways in the T-cell activation cascade, which may explain the differences in cytokine expression. Our results are in contrast to the observation of Cantorna et al, who reported that up-regulation of IL-4 and the subsequent generations of Th-2 type responses are mandatory for the immunoregulatory effects of vitamin D_3 [34]. Therefore,

further experiments shall provide a more detailed insight into the characteristics of 1,25DHC mediated immunoregulatory mechanisms and its effects on Th-1 and Th-2 related cytokine expression.

Taken together, our results indicate that 1,25DHC is a potent immunosuppressant in renal transplantation and may serve as a dose-reducing agent in CsA immunosuppressive regimen. When the hypercalcemic effects of 1,25DHC can be circumvented by a long-term low calcium diet, 1,25DHC might prove a valuable adjunct in the armamentarium of immunosuppressant agents.

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