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• BASIC RESEARCH •

Effect of 1,25-dihydroxyvitamin D3 on preventing allograft from acute rejection following rat orthotoic liver transplantation

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Abstract

AIM: To study the mechanism and the preventive role of 1, 25-dihydroxyvitamin D3 in acute rejection following orthotopic liver transplantation.

METHODS: Rats were randomly divided as donors or recipients for orthotopic liver allotransplantation model. Four groups were designed in the study, Group I: syngenic control (Wistar to Wistar); Group II: acute rejection (SD to Wistar); Group III: acute rejection treated with cyclosporine A, and Group IV: acute rejection treated with 1,25-(OH)2D3. Liver function, rejection activity index and mRNA of IFN-γ, IL-10 intragraft in recipients were measured on day 1, 5, 7, 15, 30 posttransplant for assessing graft function, severity of acute rejection and immune state of recipients.

RESULTS: Survival time of recipients in Group IV was significantly prolonged (4/6 recipients survived for over 100 days. *vs* Group II, $P<0.001$; *vs* Group III, $P>0.05$). After treatment with 1,25-(OH)2 D3, mean value of all the assay tested on each experimental time was compared, liver function in group IV was significantly improved (AST 127±41 U/L-360±104 U/L, BIL 13±5 mmol/l-38±11 mmol/l; *vs* Group II, $P<0.05$; *vs* Group III, $P>0.05$. Rejection activity index was significantly decreased (0-3.3±1.6; *vs* Group II, $P<0.05$; *vs* Group III, $P>0.05$). Level of hepatic IFN-γ mRNA in group IV was decreased, while level of hepatic IL-10 mRNA was increased (*vs* Group II, $P<0.05$; *vs* Group III, $P>0.05$).

CONCLUSION: Our results indicated that 1,25-(OH)2D3 induced the secretion of cytokine toward to Th2 type, which would alleviate acute rejection, protect liver function and prolong survival of recipient after orthotopic liver transplantation.

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INTRODUCTION

1,25-dihydroxyvitamin D3 (1,25-$(OH)_2D_3$), the functional metabolite of vitamin D, is a key regulator of calcium and phosphorus[1], has important immunomodulatory action[2,3], and was demonstrated to be able to prevent graft from acute rejection after transplantation of heart and renal, and prolong the survival of graft significantly[4-7]. In previous study, we demonstrated that 1,25-$(OH)_2D_3$ played important role in preventing the rejection of allograft after liver transplantation. The kinetic characteristic of 1,25-dihydroxyvitamin D3 on liver allograft viability and rejection after liver transplantation was explored in present study with orthotopic rat liver transplantation model. Furthermore, expression of IFN and IL-10 was determined to examine the immunomodulatory effect of 1,25-dihydroxyvitamin D3.

MATERIALS AND METHODS

Animals, surgical procedure and experimental groups

Male Sparague-Dawley (SD) and Wistar rats (200-250 g, purchased from Shanghai Animal Center, Academy of Science, Shanghai) were selected randomly as transplant donors or recipients. Under ether inhalation, orthotopic rat liver transplantation was performed according to Kamada's two-cuff technique[8]. Four experimental groups were designed in this study, Group I: syngenic control (Wistar-to-Wistar); Group II: acute rejection (SD-to-Wistar); Group III: acute rejection treated with cyclosporine A 3.0 $mg\cdot kg^{-1}\cdot d^{-1}$ intramuscularly, from day 0 to 13 posttransplant (SD-to-Wistar+CsA); Group IV: acute rejection treated with 1,25-(OH)2 D3 1.0 $\mu g\cdot kg^{-1}\cdot d^{-1}$ intraperitoneally, from day 0 to day 13 posttransplant (SD-to-Wistar+1,25-(OH)2 D3). Recipient animals had an experimental diet containing 0.47 % calcium 7 days before transplantation; only recipients in Group IV received experimental diet for15 days following transplantation.

Sample harvesting

On day 1, 5, 7, 15, and 30 posttransplant, three rats were selected from each group for sample harvesting. Serum calcium levels were measured to study the effect of 1,25-$(OH)_2D_3$ on calcium metabolism. Serum aspartate aminotransferase (AST) and total bilirubin (BIL) were measured to study the effect of 1,25-$(OH)_2D_3$ on liver functions. Liver allografts were taken for histology and cytokine determination. Another 6 rats in each group were bred for observing survival time. Rocaltrol®, 1,25-dihydroxyvitamin D3 product of Roche Pharma, and Sandimmune®, Cyclosporine A product of Novartis Pharma were used in this study.

Histopathologic examination

Grafted liver samples were fixed in 10 % buffered formalin and embed in paraffin. Five-micrometer-think sections were affixed on slides, deparaffinized, and stained with hematoxylin and eosin. Morphologic change of graft was observed and severity of acute rejection was assessed with Rejection Activity Index according to Banff 97 working classification of hepatic allograft pathology[9].

Cytokine reverse transcription-polymerase chain reaction

Primer sequences and reaction conditions The sequences of primers, synthesized by Bioengine-ering Corp at Shanghai

are as follow, IFN-γ sense primer 5' -ACT GCC AAG GCA CAC TCA TT-3' , antisense primer 5' -AGG TGC GAT TCG ATG ACA CT-3' (size 235bp); IL-10 sense primer 5' -TGC TCT TAC TGG CTG GAG TG-3' , IL-10 antisense primer 5' -GTC GCA GCT GTA TCC AGA GG-3' (size 345bp). β-actin sense primer, 5' -TCG TAC CAC TGG CAT TGT GA-3' , β-actin antisense primer, 5' -TCC TGC TTG CTG ATC CAC AT-3' (size 645bp). Amplification were performed using an initial denaturation step of 95 ℃ for 2 minutes, followed by 32 cycles consisting of 94 ℃ for 45 seconds, 56 ℃ for 45 seconds and 72 ℃ for 45 seconds. The final extension step was one cycle at 72 ℃ for 10 minutes.

RT-PCR Total RNA was prepared from grafted liver with TRIzol Reagent (Gibco, BRL) according to the manufacturer' s recommendations. For cDNA synthesis, 4 μg total RNA was reverse transcribed with MuLV (MBI, Fermentas) reverse transcriptase according to the manufacturer' s recommendations. Two microliters from the resulting cDNA solution were then amplified in a volume of 25 μl PCR buffer using specific oligonucleotides under the conditions aforementioned. Reaction products were run on a 1.5 % agarose gel for 20-30 min at 100 V, and visualized with ethidium bromide under UV light. Relative expression of cytokines was defined as optical density ratio (cytokine/β-actin) analyzed by Kodak science scanning system.

Statistics

All data were expressed as mean values and standard deviations and analyzed using SPSS software (version 10.0 for windows). Difference in mean value between the groups was tested by Independent-Samples *t* test. Differences in pathological Reject Activity Index score between the groups were tested with the Mann-Whitney U nonparametric test. Recipient' s survival was estimated with the Kaplan-Mier product limit estimator. Statistically significance was defined at $P<0.05$.

RESULTS

Survival of recipient posttransplantation

All the recipients in Group I survived for over 100 days; all the recipients in Group II died at day 7 to day 19 posttransplantation and median survival time was 12.3±4.0 days. Five out of 6 recipients in Group III, and 4 out of 6 recipients in Group IV survived for long term. Difference between Group IV and II was statistically significant, but not for that between group IV and III. Kaplan-Mier Survival Curve was showed in Figure 1.

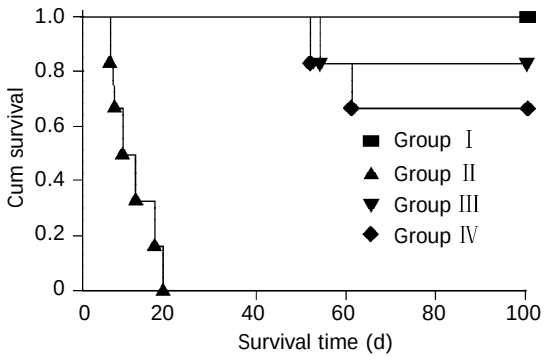

Figure 1 Effect of 1,25-$(OH)_2D_3$ on survival of rat recipients of an othtopic liver allograft (Kaplan-Meier Survival Curve). When Group III was compared with Grou II: $P=0.0005$. When Group IV was compared with Grou II: $P=0.0005$. When Group IV was compared with Group III: $P=0.70$.

Effect of 1,25-$(OH)_2D_3$ on serum calcium and liver function

An obvious limitation to the use of vitamin D3 derivatives in transplantation was hypercalcemia. Serum calcium in Group I on day 7 posttransplant was defined as basal value. If value was not significant in comparison with basal value, no significant effect of 1,25-(OH)2 D_3 or CsA on calcium metabolism was considered (Table 1). Level of AST and BIL in Group I increased slightly within 7 days posttransplant and then gradually restored to normal after 7 days posttransplant. In Group II, liver function deteriorated dramatically on day 5 posttransplant, and levels of bilirubin and AST increased steadily until the death of recipients. In contrast, administration of either CsA or 1,25-(OH)2 D_3 prevented deterioration of the graft function during the first 30 days after transplantation. The average values of AST were 146±33 U/L-241±107 U/L, and BIL 17±6 mmol/l-25±9 mmol/l in Group III, while mean level of AST, BIL in Group IV posttransplant was 127±41 U/L-360±104 U/L and 13±5 mmol/l-38±11 mmol/l, respectively. Difference of these values between Group II and IV was statistically significant while difference between group III and IV was not (Figure 2).

Table 1 Serum calcium assessment (mmol/l, $\bar{x}\pm s$)[a]

Group	Time posttransplant (d)		
	7	15	30
I	2.29±0.13	2.16±0.05	2.22±0.16
II	2.34±0.04		
III	2.25±0.11	2.32±0.07	2.12±0.09
IV	2.60±0.31	2.47±0.27	2.33±0.31

a: Serum calcium of Group I on 7 d posttransplant was supposed as basal values, each value was not significant in comparison with basal values ($P>0.05$).

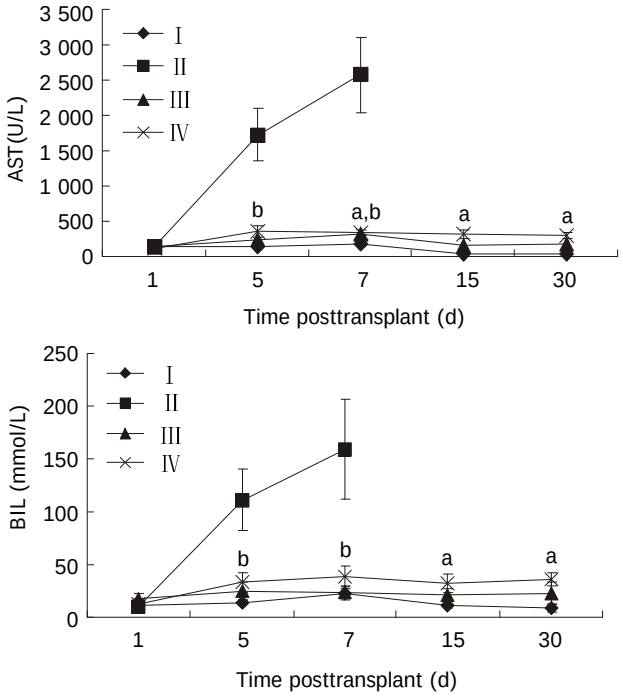

Figure 2 Effect of 1,25-$(OH)_2D_3$ on graft function ($\bar{x}\pm s$). [a]$P<0.05$, *vs* Group I; [b]$P<0.05$, *vs* Group II.

Histological assessment of graft rejection

In Group I no signs of rejection were found all the time, on day 5 posttransplant, minimal inflammation on portal area was found, average RAI score was 0.3±0.6. On all other experimental times, RAI score was 0; In group II, a few lymphocytes infiltrated in portal area with minimal vein endothelialitis on day 1 posttransplant. Lymphocytes infiltrated

in portal area obviously with degeneration of hepatic parenchyma in all cases on day 5 posttransplant with average RAI 8.3±1.1 Marked mononuclear infiltration, severe vein subendothelialitis with bridging hepatocellular necrosis can be found on day 7 posttransplant with average RAI 8.7±0.6. Rejection reaction was greatly inhibited in Group III due to the immunosuppressive effect of CsA. No evidence of rejection was found on day 1 and day 5 posttransplant. But both inflammatory infiltration and endothelialitis can be found on day 7 with RAI at 2.3±0.6 was evaluated. Infiltration in portal area and bile duct hyperplasia in some cases were detected on day 15 and day 30 postransplant. As for Group IV, RAI was 0 on day 1 posttransplant. On day 5 posttransplant, RAI was 2.3±0.6. Inflammatory was mild. Vein subendothelial tissue and bile duct were cuffed by lymphocytic infiltrate occasionally. Necrosis of hepatocytes was not detected. On day 7 and 15, mild to moderate portal inflammatory was continuously mild to moderate. Various degree endothelialitis or hepatocyte necrosis existed in some cases. On day 30 posttransplant, mild to moderate portal infiltrate was still existed. Mild bile duct hyperplasia was found in 2/3 cases. RAI in Group IV was lower than in Group II significantly (P<0.05) on each time point. In comparison with Group III, RAI in group IV was slightly higher without any significance (P>0.05) on each time point (Figure 3).

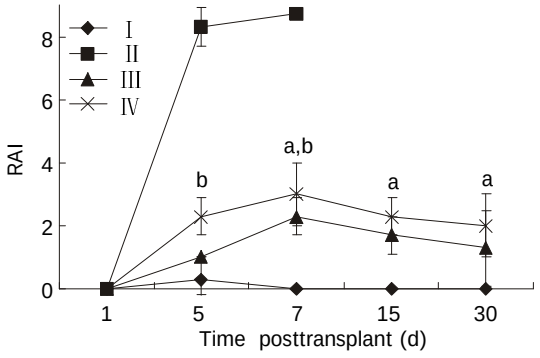

Figure 3 Effect of 1,25-$(OH)_2D_3$ on Rejection Activity Index (RAI). ($\bar{x}$±s). [a]P<0.05, *vs* Group I; [b]P<0.05 *vs* Group II.

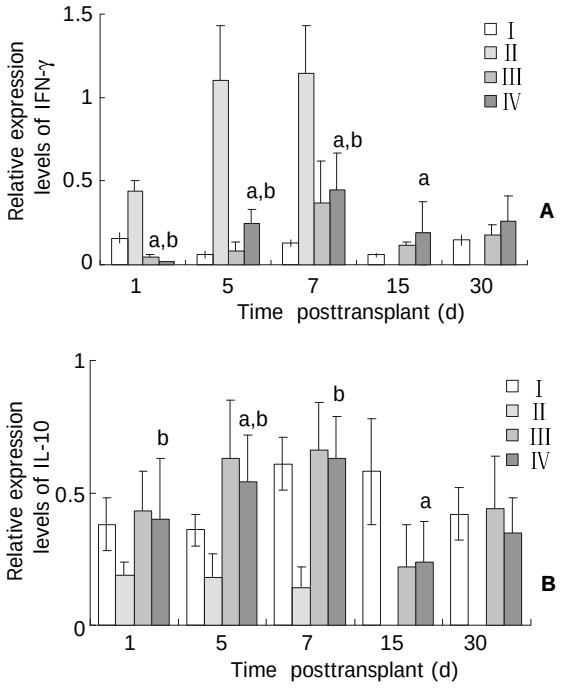

Figure 4 Effect of 1,25-$(OH)_2D_3$ on IFN-γ and IL-10 gene transcription ($\bar{x}$±s, analyzed by RT-PCR). [a]P<0.05, *vs* Group I; [b]P<0.05 *vs* Group II.

Effect of 1,25-$(OH)_2D_3$ on IFN-γ mRNA and IL-10 mRNA

On each defined time posttransplant, the expression of IFN-γ mRNA intragraft was little in Group I and strong in Group II. After administration of CsA, the expressed level of IFN-γ mRNA decreased significantly (P<0.05, *vs* Group II). After treatment with 1,25-$(OH)_2D_3$ 1.0 μg· kg^{-1}· d^{-1}, the expressed level of IFN-γ mRNA decreased significantly (P<0.05, *vs* Group I; P>0.05, *vs* Group III).

In contrast, expression of IL-10 mRNA intragraft was strong and obvious in Group, but very weak in Group II. The expression level increased significantly (P<0.05, *vs* Group II) after treatment with CsA. As for Group IV, the expression level increased markedly (P<0.05, *vs* Group II; P>0.05, *vs* Group III) (Figure 4).

DISCUSSION

As a newly recognized hormone, 1,25-$(OH)_2D_3$ has immune activity *in vitro* and its role in organ transplantation has been highlighted last decade. For instance, MC1288, a analogue of 1,25-$(OH)_2D_3$, could prolong survival of cardiac and small-bowel allografts in rats[4]. 1,25-$(OH)_2D_3$ was demonstrated to inhibit neonatal as well as vascularized heart transplantation rejection much effectively than a high-dose CsA regimen[5]. However, no effect was observed in graft survival in a neonatal nonvascularized murine heart transplantation model in another report[10]; Jordan *et al*[11] reported a marginal effect of vitamin D on rat cardiac allograft survival. In all cases, significant toxicity of hypercalcium was observed. In our study, we showed that 1,25-$(OH)_2D_3$ can effectively inhibit acute rejection following liver transplantation, and prolong recipients' survival markedly. Our study also showed that hypercalcemic effect of 1,25-$(OH)_2D_3$ can be mitigated by a low-calcium diet. The major differences between these studies were the administrative route of 1,25-$(OH)_2D_3$. It was given every other day intraperitoneally in previous study. Since the half-life of 1,25-$(OH)_2D_3$ is few hours[1], the administration of this compound every other day would not be sufficient. Furthermore, several studies used various analogues of vitamin D such as KH1060, MC1288. These analogues had varied side effect of hypercalmia by changing its stereochemistry at C-20[12-14], and allowed to take higher dosage of this agents and thus increased its immune effect in therapy.

In present study, it has been confirmed that the beneficial of 1,25-$(OH)_2D_3$ on survival was due to a marked inhibition of rejection and amelioration of graft function. At the cellular level, 1,25-$(OH)_2D_3$ interferes with function of antigen-presenting cells by decreasing MHC class II expression, and blocks mitogen stimulated T-cell proliferation[15-17]. As a result, 1,25-$(OH)_2D_3$ reduces the immunogenicity of allograft and the cytotoxicity of CTL, prevents the allograft from immune attack. In present study, the allografts of rats that did not receive 1,25-$(OH)_2D_3$ demonstrated moderate to severe acute rejection. Marked lymphocytic infiltration, severe bile duct injury, subendothelialitis and hepatic necrosis were observed. The RAI score and bilirubin concentration, AST activity increased continuously until the death of recipients. In contrast, allografts of rats receiving 1,25-$(OH)_2D_3$ showed significant improvement. Lymphocytic infiltration intragraft and hepatocellular necrosis were mild, and the rejection activity was inhibited. On each time point observed, the differences in values of RAI, BIL and AST between Group III and IV were not significant statistically. It suggested that the effect of 1, 25-$(OH)_2D_3$ and CsA in protecting graft function was equal.

Some studies[18-21] showed that in allografting Th1 cells launched rejection by priming the cytotoxicity of CTL and delayed-type hypersensitivity reaction through cytokine, and Th2 cells induced allografts tolerance by receding the activity

of Th1 cells through cytokine. 1,25-$(OH)_2 D_3$ interacted with a nuclear receptor (VDR). In nuclear, VDR combined with RXR to form a heterodimer, then bound to the target gene. Once 1, 25-$(OH)_2 D_3$ combined with the VDR, DNA bending occurs. Ultimately it affected the RNA polymerase activity for either stimulation or suppression of transcription[22-24]. The present study has demonstrated that 1,25-$(OH)_2 D_3$ can inhibit transcription of IFN-γ, and stimulate transcription of IL-10. Thus, our results provide further evidence that a high IL-10 and low IFN-γ expression state may protect allografts[25,26]. It was manifested *in vitro* that 1,25-$(OH)_2 D_3$ could inhibit interleukin 12[27] which was produced by myelomonocytic cells and played a pivotal role in the development of Th1 cells, as well as inhibition the excretion of cytokine such as IFN-γ[28,29] and IL-2[30,31]. In the other hand, 1,25-$(OH)_2 D_3$ can directly stimulate Th2 cells to excrete cytokine such as IL-4, IL-5 and IL-10[32-34]. The effect of vitamin D3 on cytokine may shift the immune response from the Th1 pathway, which leads to allograft rejection to the Th2 pathway, which can induce allograft tolerance.

In kinetic surveillance, some common characteristic can be found in all groups. In isograft, variation of each index was relatively gentle. The allografts of rats that did not take 1,25-$(OH)_2 D_3$ demonstrated a obvious tidemark of rejection on day 5 posttransplant. In Group III and IV, the rejection reaction was inhibited markedly due to the immunosuppressive effect of 1,25-$(OH)_2 D_3$ and CsA. The kinetic in the two groups and IFN-γ mRNA and RAI were similar, that is, the severe rejection reaction appeared on day 7 posttransplant, meanwhile expression of IL-10 mRNA and liver function were very low. Previous studies[35,36] showed that high immunoresponses occurred day 3 to day 5 posttransplantation and thus called a transient "rejection crisis". It may be due to the strong immunosuppressive effect of 1,25-$(OH)_2 D_3$ and CSA that rejection crisis phase in Group III and IV was postponed. Interesting, although majority of recipients in group III and IV survived for long-term, all of grafts in these two groups were demonstrated various degree of rejection activity. Further studies[37,38] have confirmed that majority of this rejection was self-limited, and it could resolve spontaneously by day 50 posttransplant without immunosuppressive agents.

In conclusion, our study proved that 1,25-$(OH)_2 D_3$ could effectively modulate the cytokine net, induce TH1/TH2 shifting, and thus postpone the "rejection crisis", inhibit the acute rejection and protect the graft function.

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