

Strong additive effect of 1,25-dihydroxycholecalciferol and cyclosporine A but not tacrolimus in rat lung allotransplantation[☆]

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Received 27 September 2002; received in revised form 9 April 2003; accepted 26 April 2003

Abstract

Objectives: 1,25-Dihydroxycholecalciferol (calcitriol, vitamin D3) has immunosuppressive properties. This study evaluates the effect of calcitriol in combination with either cyclosporine A or tacrolimus on acute lung allograft rejection in a rat model of unilateral left lung allotransplantation. **Methods:** Unilateral left lung transplantation was performed in male rats (Brown–Norway to Fischer F344, 200–250 g body weight). For immunosuppression, the following subtherapeutic doses were used: calcitriol 0.5 µg/kg/day, cyclosporine A 2.5 mg/kg/day i.p., and tacrolimus 40 µg/kg i.m. Five groups ($n = 5$) were analyzed: cyclosporine A; cyclosporine A and calcitriol; calcitriol; tacrolimus and calcitriol; and tacrolimus. The injections were performed for 5 days starting from the day of transplantation. Recipients were sacrificed on day 5 post-transplant. The contralateral right main bronchus and pulmonary artery were occluded for 5 min and blood was drawn for blood gas analysis. The grafts were excised, fixed in formaline and embedded in paraffin. Histological evaluation was done in blinded fashion (ISHLT 1999/rank scale). The mean and standard error of the mean (PaO₂) or the median and range (rejection grading) are given. ANOVA followed by planned comparison for the PaO₂ and Kruskal–Wallis ANOVA for rejection grading were applied, $p < 0.05$ considered significant. **Results:** Arterial PaO₂ on day 5 was very low in animals treated with subtherapeutic dosages of either cyclosporine A (48 ± 10 mmHg), calcitriol (51 ± 3) or tacrolimus (86 ± 22). Combined treatment with cyclosporine A and calcitriol revealed a significant improvement (248 ± 78 ; $p < 0.05$ vs. other groups), whereas the combination of tacrolimus with calcitriol did not reveal any benefit (65 ± 9). Rejection grading with these subtherapeutic doses did not show any significant difference between groups. **Conclusions:** Our data indicate that cyclosporine A, but not tacrolimus, has a strong additive effect with calcitriol on acute rat lung allograft rejection.

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Keywords: Lung transplantation; Immunosuppression; Vitamin D3; Cyclosporine A

1. Introduction

Lung transplantation is an established therapeutic option in end-stage pulmonary disease. However, either acute or chronic rejection episodes limit the long-term survival of lung transplant recipients. Conventional immunosuppressive drugs such as cyclosporine A exert their effect at doses close to the toxic range. In addition, the high level of

immunosuppression as compared to other solid organ transplant recipients causes numerous side effects of the different drugs. The addition of new immunosuppressive substances may help to reduce the dose of the established immunosuppressive drugs and therefore reduce toxic side effects, e.g. nephrotoxicity of cyclosporine A.

A substantial effect of the active form of vitamin D3, calcitriol, on acute rejection has been observed in different models of solid organ transplantation [1–3], however, clinical use of calcitriol in high doses is hindered by development of hypercalcemia and hypercalcuria [4]. Therefore, this study evaluated the effect of subtherapeutic doses of calcitriol in combination with two clinically

[☆] Presented at the 16th Annual Meeting of the European Association for Cardio-thoracic Surgery, Monte Carlo, Monaco, September 22–25, 2002.

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established immunotherapeutic drugs on acute rejection in a major mismatched orthotopic rat lung allotransplantation model.

2. Methods

2.1. Experimental groups

A model of allogeneic left lung allotransplantation in rats with one major and one minor immunological mismatch (Donor Brown–Norway; Recipient Fischer F344; obtained from Harlan Netherlands B.V., Ad Horst, Netherlands) was applied. Animals received conventional food ad libitum. No specific low calcium diet was applied. Five groups ($n = 5/\text{group}$) were analyzed: I. cyclosporine A, II. cyclosporine A and ViD3, III. ViD3, IV. tacrolimus and ViD3, V. tacrolimus. For immunosuppression, the following subtherapeutic doses were used: ViD3 0.5 $\mu\text{g}/\text{kg}/\text{day}$ i.p., cyclosporine A 2.5 $\text{mg}/\text{kg}/\text{day}$ i.p., and tacrolimus 40 $\mu\text{g}/\text{kg}/\text{day}$ i.m. The injections were performed until sacrifice after 5 days starting from the day of transplantation.

Calcitriol (Rocaltrol[®]) 10 ml solution, 1 $\mu\text{g}/\text{ml}$, was provided by Roche, Basel, Switzerland. Cyclosporine A (Sandimmun[®]) was provided by Novartis Pharma AG, Basel, Switzerland and diluted in castor oil in a ratio of 1:9, and tacrolimus (Prograf[®]) was obtained from Fujisawa GmbH, Munich, Germany.

All animals received humane care in compliance with the 'Principles of Laboratory Animal Care', formulated by the National Society for Medical Research, and the 'Guide for the Care and Use of Laboratory Animals', prepared by the Institute of Laboratory Animal Research and published by the National Institutes of Health (NIH Pub. No. 86-23, revised 1985). The protocol was approved by the local animal study committee.

2.2. Operative procedure

2.2.1. Donor

The animal is anaesthetized in a glass chamber by inhaling 4% Halothane (SIGMA, Buchs, Switzerland). Thiopental (Penthotal[®], Abbott AG, Baar, Switzerland) at a dosage of 50 mg/kg body weight is injected i.p. Heparine (Liquemin[®], Roche Pharma, Reinach, Switzerland) is administered by injection into the penile vein (500 IU/kg body weight). A tracheostomy is performed and the animal is ventilated with a 14 GA catheter (Insyte[®], Becton Dickinson, Sandy, UT, USA) with 100% oxygen, a breathing frequency of 100/min and a tidal volume of 10 ml/kg body weight by a Harvard rodent ventilator (Model 683, Harvard Apparatus, South Natick, MA, USA). After cutting the inferior vena cava and left appendix of the heart, a small silicon hose is inserted into the main pulmonary artery (PA) via an incision in the right ventricle.

Both lungs are flushed with 20 ml of LPD solution (Perfadex[®], Medisan Pharmaceuticals, Uppsala, Sweden) at a pressure of 20 cm H₂O. The trachea is then tied with the lungs in end-inspiratory position. The heart–lung block is removed and the left lung is separated ex vivo from the heart and right lung. Small plastic cuffs are placed around the PA and vein [5], the vessels are everted and tied onto the cuff and fastened with 8-0 monofilament thread (Surgipro[®], USSC, Norwalk, CT, USA). The lung is stored in LPD solution at 10 °C until implantation.

2.2.2. Recipient

The recipient is anaesthetized by breathing 4% Halothane in a glass chamber, intubated, and anaesthesia is maintained with Halothane. A left thoracotomy is performed in the fourth intercostal space. The left hilum is dissected, and removable microvascular clips are put onto the left PA and left pulmonary vein (PV). The left main bronchus is ligated with 6-0 polyfilament thread (Sofsilik[®], USSC, Norwalk, CT, USA) and cut off. An incision is made in both PV and PA. The vessels are flushed with heparinized saline solution. The cuffs of the donor lung are inserted into the recipient's vessels, and 6-0 polyfilament ligatures (Sofsilik[®]) are placed around the cuffs and tied. The native PA and PV are cut off beyond the anastomosis and the native lung is removed. A 9-0 monofilament running over-and-over continuous suture (Monosof[®], Tyco Healthcare, Wollerau, Switzerland) is employed for bronchial anastomosis. Ventilation and then retrograde followed by antero-graduate perfusion of the graft are restored by removing the clips from left bronchus, PV and PA, respectively. A small chest drain is inserted to the left hemithorax, and the thoracotomy is closed with four layers of continuous suture. The chest drainage is removed after the animal restores spontaneous breathing, and the animal is extubated.

2.3. Statistics

For continuous data (arterial oxygen content), all values are given as mean \pm standard error of the mean. Rejection grading is given as the median and range.

As PaO₂ values did not fulfill homogeneity criteria of variances, ANOVA followed by planned comparison with contrast vector analysis was performed on log-transformed data. The comparison of rejection grading was done by the non-parametric Kruskal–Wallis ANOVA.

2.4. Assessment

2.4.1. Graft function

Five days after transplantation, the recipient animal is preanaesthetized in a glass chamber inhaling 4% Halothane. Thiopental (50 mg/kg body weight) is injected i.p. The animal is ventilated via a tracheostomy by a Harvard Rodent Ventilator with 100% oxygen, a breathing frequency of 100/min and a tidal volume of 8 ml/kg.

A thoraco-laparotomy in the anterior midline is performed. Microvascular clips are put on the right main bronchus and right PA in order to ventilate and perfuse only the isolated left lung graft. Five minutes after occlusion, a steady state is reached and 300 μ l of blood is aspirated from the aortic arch to a syringe (Radiometer Pico™ 50, Copenhagen, Denmark) for blood gas assessment (Radiometer ABL 700 Serie, Copenhagen, Denmark). Subsequently, the inferior vena cava and left appendix of the heart are incised and a small silicon hose is inserted into the main PA via an incision in the right ventricle. The lungs are then flushed with 20 ml of 0.9% saline solution under a pressure of 20 cm H₂O. The heart–lung block is explanted and tissue samples are put in 10% formaline solution (SIGMA, Buchs, Switzerland) for histological analysis.

2.4.2. Histology

The histological assessment was done by a trained lung pathologist in blinded fashion according to the Working Formulation for the Classification of Pulmonary Allograft Rejection of the International Society for Heart and Lung Transplantation [6].

3. Results

3.1. Characterisation of experimental groups

Donor and recipient rats weighed 250 ± 20 g, with no statistical difference between groups. The entire transplantation procedure (donor lung explantation, ex vivo preparation and implantation) took a mean of 126 ± 10 min. Warm ischaemic time was 21 ± 3 min, without statistical difference between groups. Ten recipients died due to technical failures, and additional transplantations were carried out to replace these animals.

3.2. Graft function

The results of blood gas analysis are summarized in Fig. 1. Overall ANOVA revealed a statistically significant difference between groups ($P = 0.004$). Combined treatment with cyclosporine A and calcitriol resulted in a significant improvement of graft function 5 days post-transplant (248 ± 78 mmHg; $p = 0.0002$ vs. all other groups), as compared to single treatment with either cyclosporine A (48 ± 10 ; $p = 0.00003$), calcitriol (51 ± 3 ; $p = 0.001$) or tacrolimus (86 ± 22 ; $p = 0.003$). Interestingly, the combination of subtherapeutic doses of tacrolimus and calcitriol did not reveal any benefit (65 ± 9 ; $p = 0.4$ vs. all other groups; $p = 0.4$ vs. calcitriol group).

3.3. Rejection grading

The histopathological assessment (Table 1) demonstrated no significant difference between the groups despite

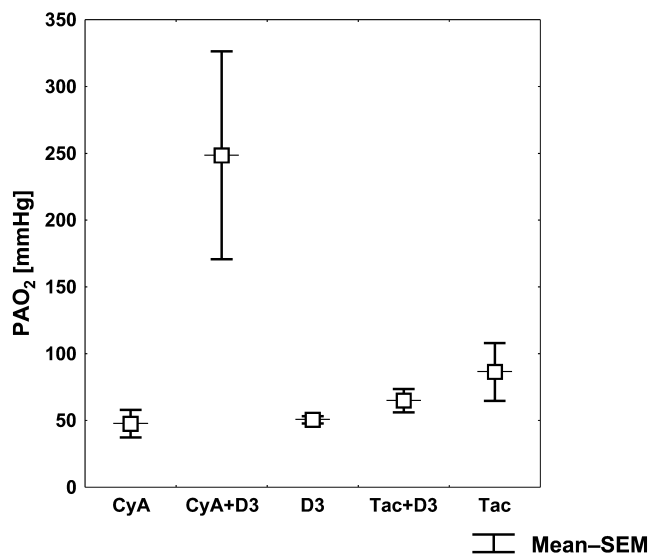


Fig. 1. Significant improvement of arterial oxygen content by combined treatment with calcitriol and cyclosporine A.

significant differences in graft function (overall group comparison for perivascular rejection $p = 0.76$; for peribronchial rejection $p = 0.11$). In addition, group comparison by the Mann–Witney U test even without Bonferroni correction for multiple testing did not reveal any significant differences.

4. Discussion

This study demonstrated a significant improvement of graft function, as assessed by arterial oxygen content, by a combined treatment with subtherapeutic doses of calcitriol and cyclosporine A 5 days after left lung allotransplantation, whereas a combination of calcitriol with tacrolimus did not reveal any protection against rejection. No beneficial effect of single treatment with one of the established immunosuppressive agents or with calcitriol at these low doses was observed.

The sterol hormone 1,25-dihydroxycholecalciferol (1,25-dihydroxyvitamin D₃, calcitriol), the active form of vitamin D₃, was considered to be a regulator of calcium, phosphorus and bone metabolism only in mammals, cholecalciferol is hydroxylated on C-25 in the liver and on C-1 in the kidneys until the late 1970s [7]. During the past, additional effects on the differentiation of hematopoietic cells, secretion of prolactin and insulin and differentiation of epidermal cells have been elucidated, and the discovery of vitamin D receptors (VDR) in a variety of cancer cells finally led to experimental applications in the treatment of carcinoma by induction of p53-independent apoptosis. In the 1980s, the immunoregulatory properties of calcitriol have been discovered and it has been shown that calcitriol suppresses T-helper cell mediated delayed hypersensitivity. Further

Table 1
Rejection grading according to the ISHLT

	CyA	CyA + ViD3	ViD3	Tac + ViD3	Tac
Vascular	A3 (A3–A4)	A3 (A2–A3)	A3 (A3–A4)	A2 (A2–A4)	A3 (A2–A3)
Airway	B3 (B2–B4)	B3 (B2–B4)	B4 (B3–B4)	B3 (B2–B3)	B3 (B2–B3)

No significant differences between groups were observed.

research in the field of autoimmune diseases was very promising. The *in vivo* effectiveness of calcitriol treatment in lupus nephritis in mice [8], Heymann's nephritis [9], experimental autoimmune thyroiditis and an experimental model of arthritis [10] led to successful application in solid organ transplantation models [2,3]. The immunosuppressive properties of calcitriol are mediated by cells possessing the nuclear VDR [7]. VDRs are present not only in the intestine, bone and kidney, but also in islet cells of the pancreas, in the parathyroid gland, hematopoietic cells, promyelocytes, activated lymphocytes, T-cells of thymus, keratinocytes of reproductive organs, and skin. They regulate transcription similarly to other members of steroid–thyroid hormone superfamily [7]. The VDR binds to the DR-3 promoter region of target genes to stimulate or suppress transcription, followed by translation of characteristic proteins. Calcitriol stimulates the cell to differentiate into the final form and then stops further proliferation (e.g. promyelocytes into monocytes).

In the field of transplantation, the effect of calcitriol on a subtype of antigen-presenting cells, in particular dendritic cells (DCs), seems to be of highest importance. These DCs undergo a process of maturation after the acquisition of antigen, which results in the upregulation of MHC and costimulatory ligands on the cell surface and in secretion of immunomodulatory cytokines. In this mature state, DCs are primed to activate T-cells in an antigen-specific fashion. The addition of calcitriol to cell cultures of murine bone marrow results in inhibited DC maturation as assessed in reduction of MHC II levels and costimulatory ligands as CD40 and B7-1 and B7-2, whereas calcitriol and also analogs of vitamin D3 did not have any effect on bone marrow cultures of VDR-deficient mice without attenuation of the number of DCs [11].

In addition, calcitriol suppresses m-RNA levels of IL-2 and IFN- γ , lymphokines produced by Th1 cells, and it has been shown that serum levels of IL-2 were lower after D3 analog treatment in mice [4]. Reduced levels of IFN- γ may evoke decreased class II antigen expression on cells like lymphocytes, monocytes, and macrophages. Other authors suggested that calcitriol is inhibiting the production of IL-12, which induces differentiation and activation of Th1 and NK cells by antigen presenting. In allotransplantation, NK cells recognize the decrease of MHC antigens and mediate target cell death by perforine, TNF- β , granzines, and by antibody dependent cell mediated cytotoxicity. On the other hand, IL-2 activates CD8 + T lymphocytes, which

show cytotoxicity in the context of class I MHC antigens. Calcitriol influences not only the T-cell lymphokine production, but also T-cell proliferation, as it arrests the cell in the G0/G1 transition phase [12]. Calcitriol also blocks IL-2 transcription mediated by nuclear factor of activated T-cell (NF-AT) [13] and interacts with the T-cell activation cascade at a level downstream calcineurin [14].

The application of a high dose treatment with calcitriol in humans, however, is not feasible due to development of hypercalcemia and hypercalcuria. Therefore, analogs of vitamin D3 with a much less calcemic effect have been developed, and applied successfully in allograft transplantation models, however, even these analogs induce hypercalcemia during long-term treatment [4]. Therefore, this study was conducted with calcitriol at a low subtherapeutic dose. In addition, calcitriol is often given already to lung transplant recipients as prophylaxis against osteoporosis induced by cyclosporine A and corticosteroids.

Whether the effect of combined treatment with cyclosporine A and calcitriol is additive or synergistic in this model, remains speculative. The fact that cyclosporine A also inhibits production of IL-2, but has a different downstream pathway via association of its drug–receptor complex with calcineurin, resulting in inhibition of T-cell activation gene transcription, may suggest a synergistic effect of both substances. In addition, it has been shown that the drug LS-2616 (Linomide) can completely counteract the immunosuppressive effect of cyclosporine A but not of an analog of calcitriol in a model of cardiac allograft survival [1], and in a model of phytohemagglutinin A-induced lymphocyte proliferation. In the latter study, a clear synergism between both substances has been demonstrated by median effect principle analysis and confirmed in a murine model for multiple sclerosis [15].

The combined treatment with tacrolimus and calcitriol revealed no benefit at all. This may be due to an insufficient dose of tacrolimus, since tacrolimus and cyclosporin A share similar mechanism of action at the molecular level by inhibition of the phosphorylase activity of calcineurin and therefore, the same effect in combination with calcitriol should be observed. On the other hand, to our knowledge, only one study demonstrated any effect of a combined treatment with calcitriol and tacrolimus on *in vitro* T-cell proliferation and cytokine production induced by human mixed lymphocyte reaction [16], and the dose-reducing effect of subtherapeutic calcitriol treatment was only fivefold in tacrolimus but 10-fold for cyclosporine A,

suggesting that despite comparable mechanism of action, these two immunosuppressants differ in their additive effect with calcitriol.

We were surprised that despite significant improvement in the group receiving combined subtherapeutic treatment with cyclosporine A and calcitriol, no difference regarding rejection grading was observed. Therefore, all slides were re-analyzed by another expert lung pathologist (data not shown). No differences regarding perivascular grading, and only very minor non-significant differences regarding peribronchial grading were observed. On the other hand, it is known that histological signs of allograft rejection in rats occur very early and continue more rapidly as compared to canine, baboons and humans, with a massive influx of leucocytes from the second day onward [17]. In another study by our group, arterial blood gas analysis at day 3 in the same strain combination used here reveals a preserved graft function with a mean PaO₂ of about 230 mmHg despite significant signs of rejection [18]. We therefore speculate that in rats, signs of rejection may occur without significant impact of rejection on allograft function, questioning the validity of the ISHLT scoring for rodents at least in early and minor rejection.

In conclusion, calcitriol can be considered a new experimental immunosuppressant, improving lung allograft function in combination with cyclosporine A even at subtherapeutic doses. Further studies are needed to confirm these results with less hypercalcemic analogs of calcitriol which may be feasible for long-term application in human lung transplant recipients.

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Appendix A. Conference discussion

Dr C. Yankah (Berlin, Germany): My first question is about your experimental design. How long was the rejection time in your control group?

Dr Stammberger: We assessed the animals 5 days after transplantation, as it is well known in this strain combination that you'll always see a complete rejection of the allograft after 5 days.

Dr Yankah: Can you describe the pharmacokinetics of the cyclosporine in the rats? Because there might be fluctuations in the blood levels of the cyclosporine in the rats which might then also influence the degree of Interleukin-2 production and T-lymphocyte activation, depending on the mode of application. Did you observe such variations in the CsA levels in your series?

Dr Stammberger: We used a preparation which is also used in humans, Sandimmune, and we applied it via intraperitoneal injection. However, we did not take any blood samples and check for levels of cyclosporine A, so I can't focus on variations in the CsA levels.

Dr C. Maurus (Zurich, Switzerland): Do you have any idea about the mechanism of the additive effect of cyclosporine and vitamin D-3?

Dr Stammberger: It is well known that both substances reduce production of IL-2. In addition, vitamin D-3 reduces IL-12 production. It is, however, suggested by recent publications that the main effect of vitamin D-3 is impairment of the maturation of dendritic cells and their ability to activate T-lymphocytes. So there are different downstream pathways between vitamin D-3 and cyclosporine A.

Dr Maurus: Regarding the reduction of IL-12: is there an effect on natural killer cells?

Dr Stammberger: Yes, there is also an effect on natural killer cell activity, that's right.