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Synergism of a natural plant product, oleanolic acid with calcineurin inhibitor in prolonging islet allograft survival

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ABSTRACT

Background: Oleanolic acid (OA) is a natural plant-derived triterpenoid with potent anti-inflammatory properties. Since inflammatory cytokines released following islet transplantation hinders engraftment and long-term function, we determined the synergistic ability of OA to with Cyclosporine-A (CSA), a calcineurin inhibitor in improving islet allograft's function and survival.

Methods: C57BL/6 mice were rendered diabetic using streptozotocin (200 mg/kg). BALB/c islets were transplanted under the kidney capsule alone (control) or along with administration of OA alone, CSA alone or a combination of both OA and CSA (OA + CSA). T-cell infiltration was analyzed by immunohistochemistry; cytokine concentration was analyzed by Luminex; T-cell cytokine phenotype was analyzed by ELISpot; and alloimmune response was analyzed by flow cytometry.

Results: OA + CSA markedly prolonged islet allograft survival compared to controls (37 ± 5 days *vs.* 8 ± 3 days). A significant decrease of CD4+ (34 ± 9 *vs.* 154 ± 42 cells/hpf) and CD8+ T-cellular (46 ± 22 *vs.* 224 ± 51 cells/hpf, $p < 0.0001$) infiltration into the graft in OA + CSA treated mice compared to controls. A significant decrease in T cell infiltration was demonstrated in the OA + CSA cohort over either compound application individually. An increase in anti-inflammatory molecules, IL-10 (2.4-fold) and vascular endothelial growth factor (1.6-fold), along with decreased pro-inflammatory cytokines IFN-γ, IL-1β (1.3–2.4-fold) and IL-17 (3.2-fold) was demonstrated. OA + CSA also significantly decreased the frequency of allo-specific T-cell responses. Development of antibodies against donor antigens was also delayed (39 vs 22 days; $p < 0.05$) in the OA + CSA cohort over administration of either agent individually.

Conclusions: OA and CSA exert synergistic effect towards enhancing islet allograft survival and function. This synergistic effect resulted in markedly reduced graft infiltrating cells with attenuation of inflammatory cytokine milieu leading to impairment of both cellular and humoral alloimmune responses. Therefore, novel therapeutic approaches involving combination of OA with calcineurin-inhibitor based immunosuppressant CSA will produce potential beneficial outcomes in clinical islet transplantation.

1. Introduction

Islet cell transplantation (ITx) offers a potential therapeutic option to patients with type-1 diabetes mellitus [1–3]. However, following transplantation, islets undergo inflammatory and allo-immune mediated-damage resulting in poor engraftment [4–7]. Pro-inflammatory cytokines, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-1β, produced by infiltrating T-cells, neutrophils and macrophages can lead to generation of free radicals within transplanted islets and thus damage the islet allografts [8–11]. In a previous study, using a subcapsular murine model of ITx, we demonstrated that treating ITx recipients with oleanolic acid (OA), prevented early islet cell loss by reducing inflammation and free-radical generation from graft infiltrating macrophages [12].

Cyclosporine-A (CSA), a calcineurin inhibitor, has been employed as an immunosuppressive agent for the prevention of rejection [13,14]. However, long term use of CSA is often associated with nephrotoxicity and also detrimental to pancreatic β-cell function [15]. Hence, immunosuppressive protocols that can substantially reduce the dose of calcineurin inhibitors with little or no deleterious effect on islet function have a significant positive impact on clinical islet transplantation.

Several *in vitro* studies have demonstrated potent anti-inflammatory effects of OA that have been attributed to the inhibition of lipoxygenase and cyclooxygenase activity, thus reducing inflammation induced by

Abbreviations: CSA, Cyclosporine-A; IP-10, inducible protein-10; ITx, islet cell transplantation; MCP-1, monocyte chemotactic protein-1; NFBG, non-fasting blood glucose; OA, oleanolic acid.

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the products of arachidonic acid degradation [16,17]. In addition, OA is a potent inhibitor of phospholipase A_2, which cleaves plasma membranes to release arachidonic acid, a substrate for inflammatory molecules including prostaglandins and leukotrienes [17,18]. OA also exerts an immunosuppressive effect by inhibiting elastases and C3-convertase of the complement activating arm of immunity [19]. Based on our previous results that OA administration *in vivo* following ITx reduced early inflammatory events resulting in improved islet allograft survival, we hypothesized that OA will synergize with currently employed low-dose calcineurin based immunosuppression. In this report, we present evidence that administration of OA with low dose CSA induces early engraftment of transplanted islets, along with reducing inflammation and prevention of allo-immune responses and thus promoting islet graft survival.

2. Objectives

The aim of this study was to investigate the synergetic anti-inflammatory and immunosuppressive effects of OA with low-dose CSA in a murine model of ITx. We hypothesize that OA can synergize with currently employed low-dose calcineurin based immunosuppressant CSA, thus acting as an efficacious immunosuppressive combination to promote allograft survival and function.

3. Materials and methods

3.1. Animals, drugs and induction of diabetes

C57BL/6 ($H2^b$) and BALB/c ($H2^d$) mice (6–8 weeks old, Jackson Laboratories) were utilized and animal studies were performed in accordance with the guidelines of the Animal Studies Committee, Washington University. The OA stock solution was made in DMSO (Sigma, St. Louis, MO), by constant stirring in a hot water bath (50 °C) followed by quick spin and sonication. The CSA stock solution was made using PBS. Streptozotocin (200 mg/kg), was administered intraperitoneally (i.p.) to induce diabetes in C57BL/6 mice. Non-fasting blood glucose (NFBG) was measured on whole blood samples obtained from the tail vein (Contour, Bayer Health, Mishawaka, IN). Mice with two consecutive NFBG ≥ 400 mg/dL were considered diabetic.

3.2. Isolation of mouse islets, transplantation and treatment

Islets from murine pancreata were isolated by collagenase digestion and transplanted under the kidney capsule as described earlier [12]. The reversal of diabetes was defined as a reduction in consecutive NFBG < 200 mg/dL post-ITx, and rejection was defined as an increase in NFBG > 250 mg/dL. The animals were administered i.p. with either DMSO-PBS (control, n = 10) or OA (25 mg/kg, n = 10) or CSA (low-dose, 25 mg/kg, n = 10) or a combination of OA and low-dose CSA (both 25 mg/kg individually, n = 15) from day −1 and daily thereafter.

3.3. Determination of serum insulin, chemokines and cytokines

The serum concentrations of insulin were measured using an insulin ELISA kit (Mercodia Inc.; Winston Salem,NC) as per the manufacturer's instructions. Serum levels of cytokines were analyzed using a multiplex bead immunoassay (Biosource International Inc.; Camarillo,CA) as per the manufacturer's protocol [20]. The concentrations of the serum cytokines were expressed in pg/ml.

3.4. Analysis of T-cell frequency

Recipient splenocytes were stimulated with irradiated donor splenocytes and the number of T-cells secreting IFN-γ, IL-10, IL-4 and IL-17 was enumerated by ELISPOT (Enzyme-linked immunosorbent spot) [21]. Briefly, Millipore MultiScreen® filter plates (Millipore Corporation, Billerica,MA) were coated overnight with 5 μg/mL capture monoclonal antibodies (mAbs) (BD Biosciences Pharmingen™; San Diego, CA). Subsequently, 3×10^5 recipient splenocytes were cultured in triplicate in the presence of irradiated donor splenocytes (1:1 ratio). After 48–72 h, appropriate secondary antibodies (Abs) were added. Following incubating with streptavidin, spots were developed and analyzed on an immunospot image analyzer.

3.5. Immunohistochemistry for graft infiltrating $CD4^+$ and $CD8^+$ T-cells

Frozen sections (6 μm) were obtained from explanted kidneys containing TxI. The sections were incubated overnight with rat anti-mouse mAbs against CD4 and CD8 T-cells (5.0 μg/mL, BD-Biosciences Pharmingen™; San Diego,CA) or isotype Ab (Chemicon International; Billerica,MA). Washed sections were treated with biotin-conjugated goat anti-rat IgG followed by streptavidin-HRP. Photomicrographs (10 fields) taken using a Nikon Eclipse 50i microscope (10×) were analyzed using morphometric software (NIS-Elements BR-3.2) by blinded observers.

3.6. Statistical analysis

Data was expressed as mean ± standard deviation. Statistical differences between means were analyzed using a paired or unpaired Student's *t*-test, or subjected to Scheffe's post-hoc test (ANOVA). Using the Scheffe's procedure all possible comparisons between the groups (Control, OA, CSA and OA + CSA) was conducted and $p < 0.05$ was considered significant.

4. Results

4.1. Combination of OA with low-dose CSA markedly improves early islet cell engraftment and survival

Following ITx, control and low-dose CSA (25 mg/kg) treated animals required 5–7 days and 7–9 days respectively to reverse hyperglycemia. However, OA (25 mg/kg) or OA + CSA (OA-25 mg/kg + CSA 25 mg/kg) treated recipients reversed hyperglycemia within 3 days following ITx (Fig. 1A). More importantly, 64% of the mice demonstrated normoglycemia by day 1 following ITx in OA and OA + CSA group in comparison to 20% in the low-dose CSA alone and none in control group (Fig. 1A).

Survival analysis demonstrated that, individual administration of OA or low dose CSA have significantly prolonged islet-allograft survival, in comparison to control (OA: 22 ± 3 days, CSA: 24 ± 4 days, vehicle control: 8 ± 3 days, $p = 0.005$ compared with vehicle control) (Fig. 1B). These results demonstrate that OA and low-dose CSA provided similar levels of immunosuppression resulting in prolongation of islet allograft survival following transplantation. Since OA is an anti-inflammatory agent and CSA is a calcineurin inhibitor based immunosuppressant we hypothesized that OA may synergize with CSA to islet survival. As shown in Fig. 1B, combined treatment (OA + CSA) further enhanced the islet allograft survival (37 ± 5 days; $p < 0.01$) compared to either OA alone or low-dose CSA alone (Fig. 1A & B). These results demonstrate that combination of OA with low-dose calcineurin inhibitor, CSA results in significant improvement in islet allograft survival. We propose that it is most likely due to the anti-inflammatory properties of OA which prevents early islet cell loss following transplantation, and thus, facilitating engraftment and long term islet function.

4.2. Combined administration of OA and low-dose CSA induced enhanced insulin expression in transplanted islet allograft

To determine the functional capability of transplanted islets, we measured serum insulin levels in mice that received ITx by ELISA and also detected the percent of insulin positive cells in the grafts after transplantation by immunohistochemistry. As shown in Fig. 2A, insulin secretion before transplant was minimal in all the groups denoting that streptozocin treatment effectively ablated islet cells. Following transplantation, increased insulin levels (0.72 ± 0.4 pg/ml) were noted from day 10 through day 20 following administration of OA alone, low-dose CSA alone or OA + CSA in comparison to the controls (Fig. 2A, $p = 0.0002$). Further, OA alone and low-dose CSA alone groups showed markedly higher numbers of insulin positive islets per high per field (ipi/hpf) histologically (OA: 14 ± 3 ipi/hpf, low-dose CSA: 19 ± 4, control: 5 ± 3 ipi/hpf, $p < 0.01$) demonstrating the presence of functionally healthy islets following ITx (Fig. 2B). It is of interest that numbers of islets which were insulin positive were noticeably higher in the OA + CSA group compared to the other three cohorts (42 ± 12 ipi/hpf, $p < 0.001$). Insulin levels in OA or CSA treated animals decreased to pre-transplant levels by day 24 indicating rejection. In contrast, the combined treatment with OA + CSA demonstrated continued high