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Immunological tolerance in a mouse model of immune-mediated liver injury induced by 16,16 dimethyl PGE2 and PGE2-containing nanoscale hydrogels

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ABSTRACT

Although immunosuppressive agents play a pivotal role in the success of organ transplantation, chronic toxicity has been a major issue for long-term treatment. The development of therapies that induce donor-specific immunological tolerance remains an important clinical challenge. In the present study, we investigated the underlying mechanisms and applications of prostaglandin (PG) E2 for the induction of immunological tolerance in mice with concanavalin A(Con A)-induced immune-mediated liver injury. The immunological tolerogenic effect of 16,16 dimethyl PGE2 (dmPGE2) pretreatment in C57B/6 male mice with Con A-induced liver injury was observed, and it was revealed that its response was partially associated with the expression of interleukin (IL)-10, an anti-inflammatory cytokine, in Kupffer cells. To apply native eicosanoids of PGE2 for tolerance induction *in vivo*, PGE2 was incorporated into L-lactic acid oligomer-grafted pullulan of an amphiphilic polymer to form a nano-sized hydrogel (PGE2-nanogel). Pharmacokinetics studies revealed that nanogel incorporation enabled PGE2 to have a prolonged lifetime in circulating blood, and a tolerogenic effect was also observed in Con A-induced liver injury, the same as with dmPGE2 pretreatment. Nanogel-based prostaglandin administration might be developed as a therapeutic agent to induce immunological tolerance, which is necessary in allogenic organ and cell transplantation.

1. Introduction

Although advanced surgical techniques and new immunosuppressive agents have been developed for organ transplantation for the treatment of life-threatening diseases, immunological rejection (both acute and chronic rejection) are still the largest problems in this field. The use of immunosuppressive agents, such as cyclosporine A (CyA) and tacrolimus, which suppress interleukin(IL)-2 expression and T cell-mediated immune responses, result in a dramatic improvement in allograft rejection. However, the number of patients suffering from drug toxicity has increased, and chronic nephrotoxity is one of the long-term complications in organ transplantation. It is therefore necessary to develop therapies to induce donor-specific immunological tolerance.

It is known that the liver favors the induction of immune tolerance rather than immunity, best illustrated by the occasional acceptance of liver allografts across the full major histocompatibility complex (MHC) barrier without any immunosuppressive agents [1]. Pre-exposure to donor cells and donor-specific soluble antigens through the portal vein of recipient animals increases their acceptance of solid tissue allografts [2], [3],. This strongly suggests that the liver plays an important role in immunological tolerance during allo-transplantation.

Recently, major progress has been made in clarifying the molecular and cellular mechanisms underlying the immunological

Abbreviations: PG, (Prostaglandin); PGE2, (Prostaglandin E2); dmPGE2 16, (16 dimethyl PGE2); PGI2, (Prostaglandin I2); Con A, (Concanavalin A); IL, (Interleukin); IFN-γ, (interferon-γ); TNF-α, (tumor necrosis factor-α); TGF-β_1, (transforming growth factor-β_1); GAPDH, (Glyceraldehyde-3-phosphate dehydrogenase); CyA, (cyclosporine A); MHC, (major histocompatibility complex); NKT, (natural killer T); KCs, (Kupffer cells); Treg, ($CD4^+CD25^+Foxp3^+$ regulatory T cells); Cox-2, (Cyclo-oxygenase-2); PBS, (phosphate-buffered saline); mAb, (monoclonal antibody); ALT, (aminotransferase); cDNA, (Complimentary DNA); NPCs, (non-parenchymal cells); FcγR, (anti-FcγR III/II); LLAo, (L-lactic acid oligomers); DDW, (double-distilled water); CDI, (N,N′- carbonyldiimidazole); Pul-g-LLAo, (LLAo-grafted pullulan); DMAP, (4-Dimethylaminopyridine); CAC, (critical aggregation concentrations); DLS, (dynamic light scattering); LC/MS/MS, (liquid chromatography/tandem mass spectrometry); SEM, (standard error of mean); ANOVA, (one-way analysis of variance).

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tolerance of the liver through the use of a murine model of concanavalin A (ConA)-induced T-cell mediated liver injury. Evidence suggests that activation of innate and adaptive immune cells, including natural killer T (NKT), Kupffer cells (KCs)/macrophages, and $CD4^+$ T cells, and their production of inflammatory cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, plays essential roles in the pathogenesis of liver injury in this model [4]. On the other hand, it has been reported that cytokines IL-6, IL-22, and IL-10, and KCs and $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) of the immune system play a critical role in inducing immunological tolerance of the liver to antigens and ameliorate Con-A-induced liver injury [5], [6].

Prostaglandin E2 (PGE2) is produced during inflammatory responses and mediates a variety of both innate and adaptive immune responses through four receptor subtypes (EP1-4) with distinct and potentially antagonistic signaling cascades. It has been demonstrated that immune-mediated organ injuries, including colon [7], lung [8], and kidney [9], are ameliorated by the administration of PGE2 derivatives. Cyclooxygenase (Cox)-2-derived endogenous prostaglandin I2 (PGI2) and PGE2 protect against Con-A-induced liver injury caused by suppression of INF-γ production in NKT cells [10]. However, the role of exogenous PGE2 administration to modulate immunological responses in the liver still remains unresolved.

The objective of this study was to investigate the possibility that exogenous PGE2 induces immunological tolerance in the liver in a Con A-induced liver injury model, based on the fact that PGE2 is a modulator of immunity in clinical applications. One of the limiting factors for the use of prostaglandins as signaling molecules is their rapid turn-over under physiological conditions; several modified PG analogs have been developed, but their clinical therapeutic application is still limited because of unanticipated side effects [11]. Therefore, it is clinically necessary to develop an administration system for the sustained effects of prostaglandins. To facilitate this, we designed a nano-sized hydrogel (nanogel) from an L-lactic acid oligomer with pullulan, a polysaccharide, and incorporated PGE2 into it. We examined the *in vivo* distribution of the PGE2-incorporated nanogel and of its effect on tolerance induction in the liver.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (8–12 weeks old) were obtained from Shimizu Laboratories Japan, Inc. (Kyoto, Japan). Measurement of plasma clearance of PGE2 was done with ICR mice obtained from Shimizu Laboratories Japan, Inc. All mice were maintained under specific pathogen-free conditions in the animal facility at Kyoto University. All experiments were conducted under a protocol approved by our institutional review board.

2.2. Animal treatments

15 mg/kg of concanavalin A (Con A) (Tokyo Biochemical Inc, Tokyo, Japan) was administered intravenously in pyrogen-free phosphate-buffered saline (PBS). Control mice were injected with PBS. 16,16 dimethyl PGE2 (dmPGE2, Sigma–Aldrich, St Louis, MO, USA), EP1/3 agonist of 17 phenyl-trinol PGE2 (Sigma–Aldrich), EP2 agonist of Butaprost (Sigma–Aldrich), EP4 agonist of CAY10580 (Sigma–Aldrich), and PGE2 (Sigma–Aldrich) were dissolved in PBS with 2.5% ethanol and administered intravenously to mice 1 h before Con A injection. To block IL-10 responses, 500 μg of anti-IL10 receptor mAb (clone 1B1.3a; bioXcell, West Lebanon, NH, USA) was injected intraperitoneally 90 min before Con A challenge. For the nanogel-based therapeutic approach, the PGE2-nanogel prepared by the following described methods was injected intravenously to mice 1 h before Con A injection. Control mice received nanogel solution without PGE2. All groups received Con A challenge 1 h after pretreatment.

2.3. Sampling of material

Mice were anesthetized lethally 24 h after Con A injection. Cardiac blood was withdrawn for plasma cytokine determination and analysis of plasma transaminases. After excision, small liver samples were frozen in liquid nitrogen for RNA isolation/reverse transcription (RT)-PCR.

2.4. Analysis of plasma aminotransferases

At 24 h after Con A administration, blood samples were obtained via cardiac puncture, immediately centrifuged at 1500 *g* for 5 min, and stored at −80 °C until analysis. Plasma ALT activities were measured using a standard clinical automatic analyzer.

2.5. Isolation of RNA and Real-time PCR for cytokine mRNAs

Total RNA was purified from liver tissue using the RNAeasy Plus Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Complimentary DNA (cDNA) was generated from 1 ng of whole RNA using a SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). cDNA was analyzed for content using SYBR-Green based, quantitative fluorescent PCR method (Applied Biosystems, Foster City, CA, USA). Fluorescence was detected with the Applied Biosystems 7500 Real-time PCR system (Applied Biosystems). The primers summarized in Table 1 were used. The following PCR conditions were used: 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for a final 10 min. GAPDH was used as a housekeeping gene. Fold-induction was calculated using the C_t method, $\Delta\Delta C_t = (C_t^{\text{target}} - C_t^{\text{housekeeping}})^{\text{infected}} - (C_t^{\text{target}} - C_t^{\text{housekeeping}})^{\text{uninfected}}$, and the final data were derived from $2^{-\Delta\Delta Ct}$.

2.6. Plasma IL10 Quantification by ELISA

Sandwich ELISA for murine plasma IL-10 was performed using an ELISA kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

2.7. Histological examination

Liver specimens were fixed in 10% buffered formalin and subsequently embedded in paraffin. Sections were stained with hematoxylin-eosin using a standard procedure and analyzed by light microscopy.

2.8. Isolation of nonparenchymal liver cells

The isolation of non-parenchymal liver cells was by the method reported by Katz et al. [12] modified as follows. Mice were euthanized and the portal vein was isolated, cannulated with a 22-guage catheter (Terumo, Tokyo, Japan), and injected with 10 ml of 1% (w/v) collagenase D (Sigma–Aldrich) in HBSS. We then mechanically disrupted the liver before a 20-min incubation in 20 ml of 1% collagenase at 37 °C. The resulting cell suspension was then passed through a 100-μm cell strainer (BD Falcon, Franklin Lakes, NJ, USA), and the cells were centrifuged at 30 *g* for 3 min

Table 1
Primers used in quantitative real-time PCR analysis.

m RNA	Forward	Reverse
IL-1β	5′-TGTAATGAAAGACGGCACACC-3′	5′-TCTTCTTTGGGTATTGCTTGG-3′
IL-2	5′-GCTGTTGATGGACCTACAGGA-3′	5′-TTCAATTCTGTGGCCTGCTT-3′
IL-4	5′-GAGAGATCATCGGCATTTTGA-3′	5′-AGCCCTACAGACGAGCTCAC-3′
IL-6	5′-TGATGGATGCTACCAAACTGG-3′	5′-TTCATGTACTCCAGGTAGCTATGG-3′
IL-10	5′-CAGAGCCACATGCTCCTAGA-3′	5′-GTCCAGCTGGTCCTTTGTTT-3′
IL-12	5′-CCAGGTGTCTTAGCCAGTCC-3′	5′-GCAGTGCAGGAATAATGTTTCA-3′
IFN-γ	5′-GGAGGAACTGGCAAAAGGAT-3′	5′-TTCAAGACTTCAAAGAGTCTGAGG-3′
TNF-α	5′-TCTTCTCATTCCTGCTTGTGG-3′	5′-GGTCTGGGCCATAGAACTGA-3′
TGF-β_1	5′-TGGAGCAACATGTGGAACTC-3′	5′-CAGCAGCCGGTTACCAAG-3′
GAPDH	5′-TGTTGAAGTCACAGGAGACAAACCT-3′	5′-AACCTGCCAAGTATGATGACATCA-3′

IL, interleukin; *IFN*-γ, interferon-γ; *TNF*-α, tumor necrosis factor-α; *TGF*-β_1, transforming growth factor-β_1; *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.