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Mizoribine—An inosine monophosphate dehydrogenase inhibitor—acts synergistically with cyclosporine A in prolonging survival of murine islet cell and heart transplants across major histocompatibility barrier ☆,☆☆

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ABSTRACT

Introduction: Mizoribine (MZR) is an inosine monophosphate dehydrogenase inhibitor. It has been widely used in Japan in the treatment of autoimmune diseases and is known to inhibit T and B cell proliferation. The aim of this study was to evaluate the efficacy of MZR as an immunosuppressive agent and determine its ability to synergize with a commonly used calcineurin inhibitor Cyclosporine A (CsA) in prolonging survival of murine islet cells and heart transplanted across major histocompatibility barrier.
Methods: Murine allogeneic islet cell transplantation between Balb/c donor mice and C57BL/6 recipient mice and heterotopic heart transplantation was done between C3H/He donor mice and Balb/c recipient mice. Recipients were divided into groups based on immunosuppression: Group 1—No immunosuppression, Group 2—MZR alone (20 mg/kg/day), Group 3—CsA alone (20 mg/kg/day), Group 4—MZR + CsA (20 mg/kg/day). Donor specific IFN-γ, IL-10, IL-2, IL-4 secreting cells were enumerated by ELISpot. Serum cytokine and chemokine concentration was measured by Luminex.
Results: Islet cell allograft recipients treated with CsA and MZR had prolonged islet function compared to other groups [normoglycemia (blood glucose <200 mg/dL) up to 32 ± 4 days, $p<0.05$]. Similarly, heart allograft survival was significantly improved in mice treated with CsA and MZR compared to other groups (50% 30-day survival, $p = 0.04$). Donor specific IFN-γ, IL-4, IL-2 secreting cells were significantly decreased in recipients treated with CsA and MZR with marked increase in IL-10 secreting cells ($p<0.05$). There was also an increase in serum IL-10 with decrease in IFN-γ, IL-4, IL-2, MCP-1, and IL-6 in mice treated with CsA and MZR
Conclusion: MZR and CsA when used in combination are potent immunosuppressive agents in murine islet cell and heart transplantation models. These agents lead to a decrease in donor specific IFN-γ with increase in IL-10 secreting cells leading to improved allograft survival and function.

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1. Introduction

Mizoribine (MZR) is an immunosuppressive agent that was initially isolated as an antibiotic from the mold *Eupenicillium brefeldianum* [1]. In its active form—mizoribine-5′-monophosphate, MZR acts as a competitive inhibitor of Inosine monophosphate dehydrogenase (IMPDH). IMPDH plays a role in the de novo synthesis of guanosine nucleotides especially in lymphocytes. Due to this MZR has a potent immunosuppressive effect on both humoral and cellular immunity [2-4]. MZR was approved for clinical use in 1984 in Japan and has been used as a immunosuppressive agent in rheumatoid arthritis [5] and a variety of autoimmune kidney diseases like lupus nephritis [6], IgA childhood nephropathy [7], steroid resistant nephritis [8] and even in renal transplantation [9]. It is also known to have anti-viral properties especially against respiratory syncytial, influenza and parainfluenza, measles and hepatitis C virus [10-12].

Cyclosporine A (CsA) is a widely used immunosuppressant that is used in both autoimmune diseases as well as in the setting of transplantation. As an inhibitor of calcineurin, it prevents the activation of both T-cells and B-cells [13]. Due to the different modes of action of MZR and CsA, we postulated that immunosuppressive properties of these two agents may synergize leading to better islet and heart allograft survival across MHC mismatches.

Abbreviations: MZR, Mizoribine; MMF, Mycophenolate mofetil; CsA, Cyclosporine; IMPDH, Inosine monophosphate dehydrogenase; NFGB, Non-fasting blood glucose.

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2. Objective

The aim of this study was to investigate the immunosuppressive effects of MZR and to determine its effect in combination with CsA in vivo models of murine allogeneic islet cell transplantation and heterotopic heart transplantation. We hypothesized that MZR can synergize with CsA thus acting as an efficacious immunosuppressive combination to promote allograft survival and function.

3. Materials and methods

3.1. Mice

Inbred 6–8 week old C3H/He ($H2^k$), male Balb/c ($H2^d$) and C57BL/6 ($H2^b$) were obtained from Jackson Laboratories, Bar Harbour, ME. All animal studies were performed in accordance with the Animal Studies Committee, Washington University, St. Louis, MO guidelines. The procedures described were done under aseptic techniques.

3.2. Induction of diabetes

Diabetes was chemically induced in C57BL/6 recipient mice using Streptozotocin (Sigma, St. Louis, MO). A single intraperitoneal injection of Streptozotocin freshly dissolved in citrate buffer at 200 mg/kg body weight dose was administered 7 days prior to transplantation. Mice with diabetes defined as two consecutive non-fasting blood glucose (NFBG) levels of greater than 300 mg/dL were used as recipients.

3.3. Murine islet cell isolation and transplantation

Islets were isolated from murine pancreata by collagenase digestion and transplanted under the kidney capsule as described previously [14,15]. Briefly, donor (Balb/c) pancreas was digested using collagenase-P (Roche, Indianapolis, IN, 2 mg/mL). Islets were purified on a Ficoll gradient and handpicked. They were cultured for 24 hours at 37 °C prior to transplantation. Approximately 500 islets were transplanted per diabetic recipient mice (C57BL/6) under the left kidney capsule. NFGB was measured daily with a cut-off of less than 200 mg/dL as a sign of graft function and rejection defined as a value greater than 250 mg/dL on 2 consecutive days.

3.4. Murine heterotopic cardiac transplantation

C3H/He ($H2^k$) cardiac allografts were transplanted heterotopically into male Balb/c ($H2^d$) mice as described previously [16]. Briefly, donor ascending aorta and the pulmonary trunk from the heart graft was anastomosed end-to-side to the recipient infrarenal abdominal aorta and inferior vena cava, respectively, using 10-0 sutures. Cold ischemic times were less than 30 min. Graft survival was checked by palpation for heart beat, direct visualization under a microscope and electrocardiogram.

3.5. Immunosuppression

CsA (Neoral, Novartis Pharmaceuticals, USA), MZR (Bredinin, Asahi Kasei Corp, Japan) and MMF (CellCept, Roche, USA) were obtained. Immunosuppressants were started immediately following transplantation as a once daily dose. CsA was diluted in normal saline and MZR and MMF in 0.2% hydroxypropyl methylcellulose and administered orally by gavage needle. The mice that were islet transplantation recipients were divided into four groups (15 islet transplantations per group): Group 1—no immunosuppression ($n=15$), Group 2 ($n=15$)—MZR (20 mg/kg/day), Group 3 ($n=15$)—CsA (20 mg/kg/day), Group 4 ($n=15$)—MZR (20 mg/kg/day) + CsA (20 mg/kg/day). Five animals from each group were sacrificed on day 7 after islet transplantation to study histology, serum for cytokine analysis and splenocytes for Enzyme Linked Immunospot Assay (ELISpot).

The immunosuppressive effect of varying doses of MZR along with CsA as well as MMF and CsA was tested in the murine heterotopic heart transplantation model. The mice were divided into seven groups (10 heart transplantations per group): Group I ($n=10$)—No immunosuppression, Group II ($n=10$)—CsA (20 mg/kg/day), Group III ($n=10$)—CsA (20 mg/kg/day) + MZR (20 mg/kg/day), Group IV ($n=10$)—CsA (20 mg/kg/day) + MZR (10 mg/kg/day), Group V ($n=10$)—CsA (20 mg/kg/day) + MMF (20 mg/kg/day), Group VI ($n=10$)—CsA (20 mg/kg/day) + MMF (10 mg/kg/day), Group VII ($n=10$)—MZR (20 mg/kg/day).

3.6. Analysis of donor specific secretion of IFN-γ, IL-2, IL-4 and IL-10 by Enzyme Linked Immunospot (ELISpot) Assay

In order to determine the frequency of cells secreting IFN-γ, IL-2, IL-4 and IL-10 in response to donor antigens, splenocytes were isolated from recipient mice (C57BL/6) on day 7 after islet transplantation. The cells were cultured in the presence irradiated donor splenocytes (Balb/c) as antigen presenting cells (APC) at 37 °C in 5% CO_2. IFN-γ, IL-2, IL-4, IL-10 ELISpot was performed as per the manufacturer's instructions (BD Biosciences, CA) with recipient splenocytes cultured in triplicate (3×10^5 cells/well) in the presence of donor APC in a ratio of 1:1. Negative control were cells cultured in medium alone (RPMI-1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 2 mM L-glutamine, 25 mM HEPES buffer, 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 μM 2-mercaptoethanol (Gibco BRL, Life Technologies)) and phytohemagglutinin was positive controls. The spots were analyzed in an Immunospot Image Analyzer (CTL, Cleveland, OH). Spots greater than +2 standard deviations of the mean obtained in the negative control wells were considered to be significantly positive and expressed as mean spots per million cells (spm).

3.7. Measurement of serum insulin by ELISA

Serum insulin concentration was measured in the islet graft recipients using Insulin quantification ELISA Kit (Mercodia Inc, Winston Salem, NC). Serum was obtained from blood collected by retro-orbital puncture on alternate days using an anticoagulant coated capillary tubes. ELISA was performed as per manufacturer's instructions. Briefly, serum samples were incubated for 2 hours at room temperature in ELISA plates precoated with monoclonal anti-insulin antibodies. The plates were washed, developed using tetramethyl benzidine substrate and read at 450 nm. Concentration of serum insulin was determined by a standard curve of the binding of a known concentration of insulin solution and expressed as pg/mL.

3.8. Measurement of serum cytokines and chemokines using luminex

Serum was obtained on day 7 after islet transplantation and the concentration of cytokines (IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40/p70, IL13, IL17) and chemokines (FGF, GM-CSF, IP-10, KC, MCP-1, MIG, MIP-1α, TNF-α and VEGF) were measured by multiplex bead assay using Luminex. Briefly, a mouse 20-plex cytokine and chemokine kit was used and assay performed as per manufacturer's instructions (Invitrogen, Carlsbad, CA). The mean fluorescence intensity of experimental and standard wells was determined on Luminex xMAP (Fischer, Pittsburgh, PA). Concentrations were obtained by a standard curve and expressed in pg/mL.

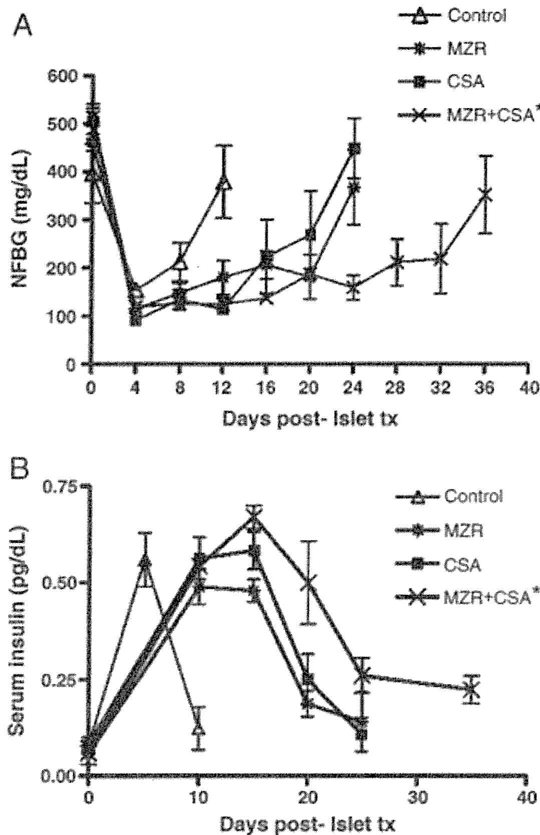

Fig. 1. Islet allograft survival and function among the recipients treated with mizoribine (MZR), cyclosporine (CsA) or a combination of both. Data is representative of mean ± SE of 10 animals/group at each time point of blood draw. *indicates $p<0.05$ in comparison to other groups. (1A) Non-fasting blood glucose measurement: Recipients treated with CsA and MZR had significantly longer (32 ± 4 days) normoglycemia (sugar <200 mg/dL) compared to other groups. Control group did not receive any immunosuppression. (1B) Serum insulin concentration following allogeneic islet transplantation.

3.9. Immunohistological analysis

Frozen samples of islet transplanted kidney were obtained on day 7 post islet transplantation and embedded in Freeze Tissue matrix (OCT), and sections 6 µm sections cut. The sections were fixed in cold alcohol for 2 min (−20 °C) and air-dried. They were treated with 3% H_2O_2 in EtOH for 10 min to block endogenous peroxidase activity followed by biotin/avidin blocking reagent for 15 min (Avidin/Biotin Blocking Kit; Vector Laboratories). Diluted primary antibodies (rat anti-mouse CD4 or CD8—5 µg/ml; BD Pharmingen) were added to sections and incubated overnight (O/N) at 4 °C. Sections were washed with tween-phosphate buffered saline and secondary antibodies added for 30 min (biotin-conjugated goat anti-rat IgG 1:50; BD Pharmingen). They were then incubated with streptavidin-HRP for 30 min at room temperature and positive cells detected by diaminobenzidine substrate kit. The sections were counterstained with hematoxylin, and examined using a light microscope. Positive cells were counted by random sampling of three areas per section. For each animals, at least 3 sections from different areas were studied and cell counts per high power field (hpf) represented as mean of 5 animals per group.

3.10. Statistical analysis

GraphPad Prism v5.0b (La Jolla, CA), SPSS v12 (SPSS Inc. Chicago, IL) and Enterprise guide v2.2 (Cary, NC) software were used for statistical analysis. Kaplan–Meier survival plots and log-rank tests were done for graft survival. Differences in drug toxicity, stimulation indices, T-cell frequencies, serum insulin levels, blood sugars and serum cytokine concentrations between the groups were compared using the Kruskal–Wallis test and two-tailed significance set at $p<0.05$.

4. Results

4.1. Significant prolongation of islet allograft survival in mice treated with CsA and MZR

Islet cells from Balb/c ($H2^d$) mice were transplanted under the kidney capsules into C57BL/6 ($H2^b$) recipient mice following induction of diabetes using Streptozotocin. Immunosuppressants were administered by oral gavage as described. NFGB was measured daily from tail vein blood and serum insulin concentration was measured on alternate days using ELISA. Control animals (Group 1, $n=10$) with no immunosuppression rejected the islet allografts by 10 ± 2 day (Fig. 1A). Administration of either MZR or CSA ($n=10$ in each group) alone prolonged islet allograft survival more than 20 days (Group 2—MZR alone—20 ± 4 days and Group 3—CsA alone 19 ± 3 days). If the animals were given both MZR and CsA (Group 4, $n=10$), the islet allograft remained functional more than 32 days (Fig. 1A). In addition, group 4 mice also demonstrated significantly longer duration of insulin secretion with detectable levels of serum insulin (0.25 pg/mL) even up to 35 days following islet transplantation (Fig. 1B).

4.2. Significant prolongation of cardiac allograft survival in mice treated with CsA and MZR

To determine whether the combination of immunosuppression using MZR and CsA will also prolong vascularized solid organ transplantation, the beneficial effects of varying doses of MZR (20 or 10 mg/kg/day) along with CsA (10 mg/kg/day) was tested using a murine heterotopic heart transplantation model. CsA alone (10 mg/kg/day), CSA with MMF (20 or 10 mg/kg/day), CsA with MZR (20 or 10 mg/kg/day) and MZR alone (20 mg/kg/day) ($n=10$ in each group) were administered following cardiac transplantation and results were compared to those that did not receive any immunosuppression. C3H/He ($H2^k$) cardiac allografts were transplanted heterotopically into male Balb/c ($H2^d$) mice and were given daily immunosuppressant by oral gavage as described above. Graft survival was checked by palpation for pulse, direct visualization and electrocardiogram. As expected, animals with no immunosuppression rejected their transplanted hearts with in 8 days following transplantation. CsA alone prolonged the

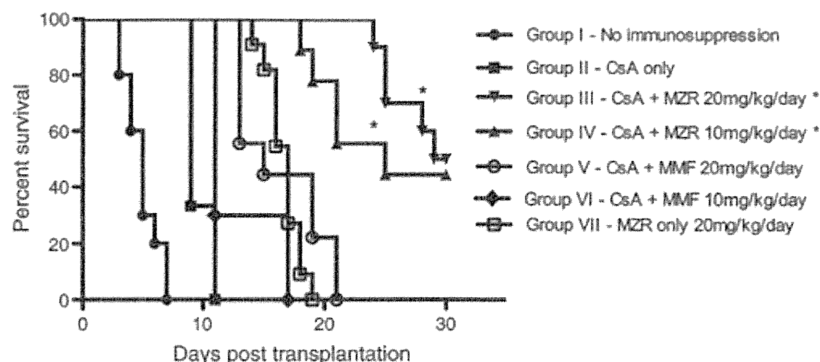

Fig. 2. Kaplan–Mier survival curve of Murine heterotopic heart allograft survival among the groups of different immunosuppressive regimens. Groups with CsA and MZR (Group III and IV) and significantly higher allograft survival (log rank $p=0.001$). Each group consisted of 10 animals each. *indicates log rank $p<0.05$ compared to other groups.

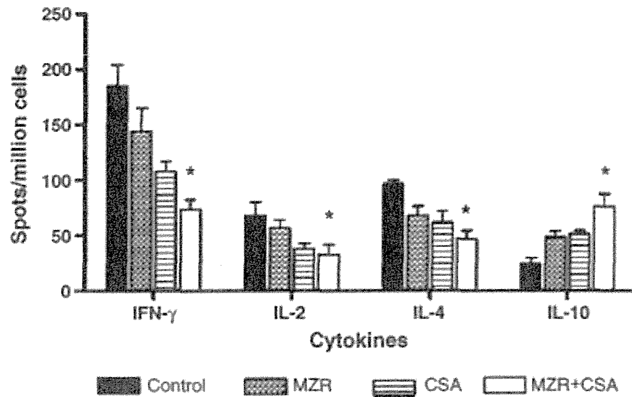

Fig. 3. ELISpot to enumerate IFN-γ, IL-10, IL-4, IL-2 secreting cells in response to irradiated donor splenocytes. Recipients treated with CsA and MZR demonstrated significantly decrease IFN-γ, IL-2, IL-4 secreting cells with increased IL-10 secreting cells in response to donor cells. Control group did not receive any immunosuppression (data expressed as mean spots/million cells ± SE). Data are representative of mean ± SE of experiments performed in triplicate with 5 animals in each group sacrificed on day 7 post islet transplant. *indicates $p<0.05$ compared to other groups.

cardiac allograft survival to 10 ± 2 days. CSA in combination with MMF prolonged cardiac allograft survival to 17 ± 2 days. In contrast, CSA in combination with MZR had a median cardiac allograft survival of 30 ± 3 days. In mice that were treated with MZR alone (group VII, $n = 10$) cardiac allograft survival were 16 ± 3 days. In brief, none of the grafts in groups I, II, V and VI were functioning at day 30 (Fig. 2). Thirty-day graft survival was the highest in the groups treated with MZR and CsA (and group III—CsA 10 mg/kg/day + MZR 20 mg/kg/day group IV—CsA 10 mg/kg/day + MZR 10 mg/kg/day) at 50% and 40% respectively (Fig. 2). It is of interest that even with lower dose of MZR (10 mg/kg/day—group IV) there was significant prolongation of cardiac allograft survival.

4.3. Decreased donor specific IFN-γ, IL-2 and IL-4 secreting cells and increased IL-10 secreting cells in islet allograft recipients treated with CsA and MZR

Donor specific responses were determined using recipient splenocytes on day 7 after islet transplantation. Cells secreting IFN-γ, IL-2, IL-4 and IL-10 were analyzed after culture with irradiated donor splenocytes and the number of cells secreting each of the cytokines was enumerated using ELISpot. As presented in Fig. 3, allograft recipients treated with a combination of CsA and MZR exhibited significantly lower donor specific IFN-γ secreting cells compared to those that did not receive immunosuppression or those who received MZR or CsA alone (in spm ± SE—180 ± 32 vs. 146 ± 26 vs. 120 ± 30 vs. 75 ± 15 spm, $p<0.05$). IL-2 and IL-4 secreting cells in response to donor antigens were also reduced in the recipients treated with CsA and MZR ($p<0.05$, Fig. 3). This was associated with an increase in frequency of donor specific IL-10 secreting cells in the mice with MZR and CsA compared to other groups (25 ± 6 vs. 50 ± 5 vs. 52 ± 3 vs. 78 ± 10 spm, $p<0.05$) (Fig. 3). The results are representative of mean in 5 animals for each group.

4.4. Decreased serum pro-inflammatory cytokines and chemokines (MCP-1, IL-2, IFN-γ, IL-6) and increased IL-10 in allograft recipients treated with CsA and MZR

Serum cytokines (IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40/p70, IL13, IL17) and chemokines (FGF, GM-CSF, IP-10, KC, MCP-1, MIG, MIP-1α, TNF-α and VEGF) concentrations were determined in islet cell allograft recipients on day 7 after islet transplantation using Luminex. In comparison to controls (no immunosuppression), those that received immunosuppression demonstrated significantly lower proinflammatory cytokine and chemokine concentration in serum (MCP-1, IL-2, IFN-γ and IL-6, $p<0.05$) (Fig. 4). More importantly, in these animals there was a significant increase in serum IL-10 ($p<0.05$) (Fig. 4) that paralleled the results obtained with increased frequency of IL10 secreting cells noted by ELISpot. In particular, the concentration of these cytokines and chemokines in those that received a combination of CsA and MZR was significantly reduced (except for IL-10 which was significantly elevated in CsA + MZR group) compared to those that did not receive immunosuppression or those who got only MZR or CsA alone: MCP-1—162 ± 15 vs. 96 ± 5 vs. 123 ± 10 vs. 85 ± 10 pg/mL; IL-2—175 ± 25 vs. 151 ± 5 vs. 75 ± 10 vs. 63 ± 10 pg/mL; IL-10—78 ± 6 vs. 131 ± 6 vs. 135 ± 8 vs. 164 ± 10 pg/mL; IFN-γ—172 ± 7 vs. 125 ± 3 vs. 119 ± 5 vs. 115 ± 4; IL-6—183 ± 20 vs. 98 ± 4 vs. 168 ± 36 vs. 100 ± 7 pg/mL (Fig. 4). The concentrations of other cytokines and chemokines measured did not differ among the groups (data not shown). All results are representative of mean of 5 animals in each group.

4.5. Decreased cellular infiltration of CD4 and CD8 cells in islet grafts in mice treated with CsA and MZR

To determine the histological differences in the islet grafts among various groups, we analyzed the infiltration of CD4 and CD8 cells in islet cells grafts on day 7 after transplantation using immunohistochemical stains. As shown in Fig. 5, treatment with CsA and MZR in combination had significantly reduced CD4 cells in comparison to untreated control, and those with CsA alone or MZR alone (CsA + MZR ($n = 5$)—4 ± 2 cells/hpf, untreated 18 ± 3, CsA alone 11 ± 4, MZR alone 12 ± 4). Similarly, mice treated with CsA and MZR had decreased CD8 cells in islet grafts on day 7 post islet transplantation compared to untreated controls and those treated with CsA or MZR alone (CsA + MZR ($n = 5$)—10 ± 3 cells/hpf, untreated 28 ± 4, CsA alone 20 ± 3, MZR alone 19 ± 3). CD4 and CD8 cell infiltration among those that either received CsA alone or MZR alone although lower than untreated control mice was not significantly different.

5. Discussion

Improvements in immunosuppressive strategies using newer and better immunosuppressive drugs have been critical to the success of both solid organ and cell transplantations across MHC barrier. This study evaluated the immunosuppressive effect of Mizoribine—an IMPDH inhibitor in murine full major histocompatibility mismatch islet cell and heterotopic heart transplantation models. We demonstrate that MZR is a potent immunosuppressive agent in promoting both islet cell as well as heart allograft survival across major histocompatibility barrier. In addition, there was a profound synergistic immunosuppressive effect for MZR when combined with CsA (Figs. 1, 2). A combination of MZR and CsA demonstrated decreased donor specific IFN-γ, IL-2, IL-4 secreting cells and increased IL-10 secreting cells seen

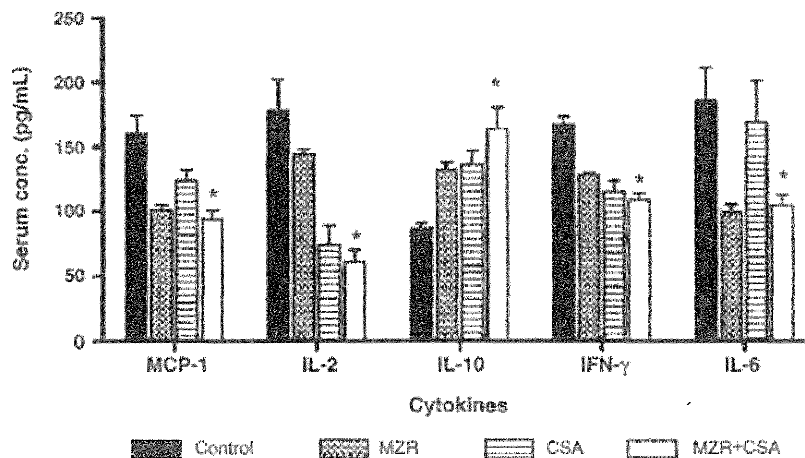

Fig. 4. Serum cytokine and chemokine concentration measured by Luminex. Recipients treated with CsA and MZR had decreased serum IFN-γ, IL-2, IL4, IL-6 and MCP-1 and increased IL-10 compared to other groups ($p<0.05$). Control group did not receive any immunosuppression (data expressed in pg/mL ± SE). Data are representative of mean of 5 animals in each group sacrificed on day 7 post islet transplant. *indicates $p<0.05$ compared to other groups.

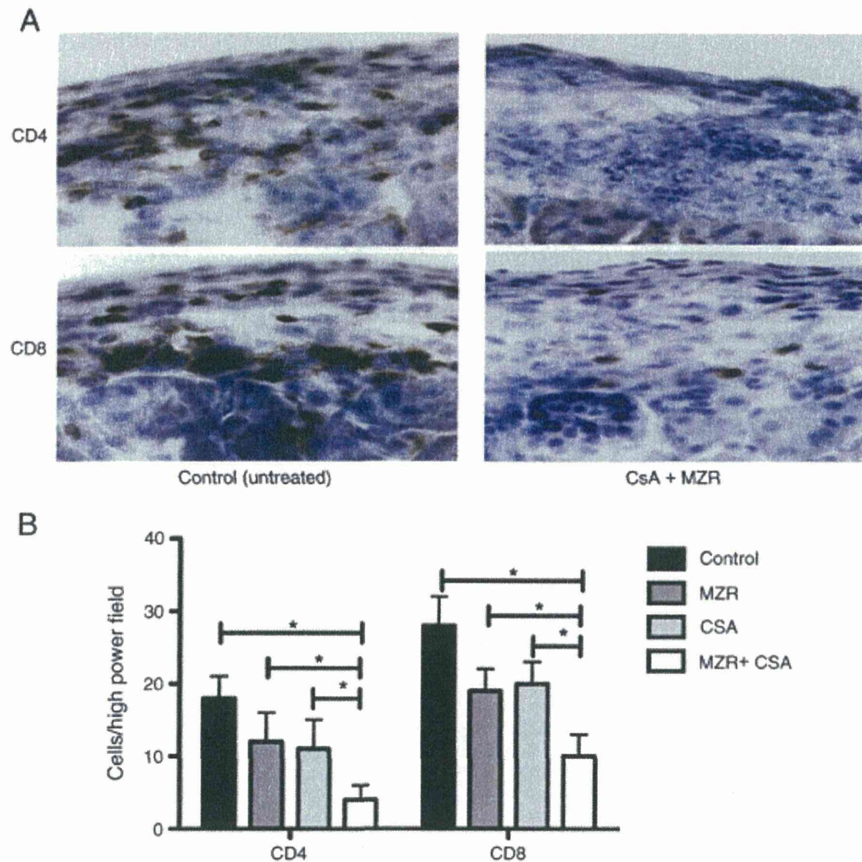

Fig. 5. Immunohistological analysis of islet cell grafts treated with different immunosuppressive drugs. Kidney from recipients of islet allo-transplantation was harvested on day 7 post-transplant. CD4 and CD8 cells were stained and number of cells/high power field were counted in 3 areas of 3 sections taken in random from islet graft recipients. Recipients with CsA and MZR treatment had significantly lesser CD4 and CD8 cell infiltration compared to other three groups. (5A) A representative high power image of CD4 and CD8 stained sections in untreated controls and those treated with MZR and CsA. (5B) Denotes mean cells/high power field of 5 islet recipients in each group. *indicates $p<0.05$.

by day 7 post transplantation (Fig. 3). This was associated with an increase in serum IL10 as well as the suppression of serum pro-inflammatory cytokines and chemokines (IFN-γ, IL-4, IL-2, IL-6, MCP-1) (Fig. 4). Also there was significantly decreased infiltration of CD4 and CD8 cells in islet graft treated with CsA and MZR in combination in comparison to untreated controls or those treated with CsA alone or MZR alone by day 7 post islet transplantation (Fig. 5). This is similar to the beneficial effect of MZR in combination with CSA reported for canine renal allograft models [17,18].

The reason for this synergistic effect of CsA and MZR can be explained by the different mechanism of action of the drugs. MZR is an inhibitor of IMPDH which is a key enzyme in the denovo purine synthesis pathway of nucleotide biosynthesis that is involved in the conversion of inosine monophosphate (IMP) to guanosine monophosphate [19]. Purine synthesis in lymphocytes primarily occurs by the denovo pathway which makes IMPDH inhibitors such as MMF and MZR an important therapeutic intervention in immunosuppression [20]. On the other hand, CsA is a calcineurin inhibitor [13]. Inhibition of calcineurin results in the blockade of translocation of the nuclear factor of activated T cells (NF-AT) into the nucleus thus inhibiting the expression of various genes involved in T cell and B-cell activation and cytokine secretion [21]. Thus due to different effects on leukocytes these collectively act as potent immunosuppressants.

There was also a difference in the immunosuppressive effect of MZR and MMF. This may be due to the different mechanisms by which MZR and MMF inhibit IMPDH. MZR acts via its active form mizoribine monophosphate, which competitively inhibits IMPDH activity by binding to the IMP binding site of IMPDH [22,23]. In contrast, MMF acts as a non-competitive reversible inhibitor of IMPDH via its active form mycophenolic acid (MPA). MPA binds to the nicotinamide adenine dinucleotide (NAD) binding site of IMPDH and thus inhibiting the conversion of IMP to xanthine monophosphate, an intermediary step in the denovo synthesis pathway [23-25]. These differential mechanisms of MMF and MZR on IMPDH inhibition also explain the synergistic immunosuppressive effects of a combination of MZR and MMF in animal models [25].

Results in this report also demonstrate marked effects of CsA and MZR in down regulating IFN-γ, IL-2 and IL-4 as well as various pro inflammatory cytokines including MCP-1 and IL-6. IFN-γ is a potent inflammatory cytokine and both CD4 and CD8 T cell mediated IFN-γ donor specific responses have been shown to play an important role in allograft rejection [26,27]. Combination of CsA and MZR suppressed the number of cells secreting IFN-γ in response to donor antigens seen by ELISpot (Fig. 3) as well as the circulating serum IFN-γ level (Fig. 4). It also decreased the CD4 and CD8 cells infiltrating the graft by day 7 following transplantation (Fig. 5). In addition, in these animals there was a concomitant increase in serum IL-10 levels along with an increase in the donor specific IL-10 secreting cells. IL-10 has been reported to be a potent anti-inflammatory cytokine and can also suppress IFN-γ secretion [28,29]. A limitation of our study is that we did not specifically select T-cells to perform ELISpot experiments to demonstrate that this was indeed T-cell mediated. However, the data from histological analysis with significant differences in CD4 and CD8 cell infiltration strongly suggests that the immunosuppressive drugs predominantly affects T cells in reducing the IFN-γ response. Thus the immunological changes that lead to a suppression of IFN-γ as well as other pro-inflammatory cytokines and chemokines (MCP-1, IL-4, IL-2) and an increase in IL-10 in the animals treated with a combination of CsA and MZR provides a

possible mechanism for the noted improvement in both islet cell and cardiac allograft survival.

In conclusion, this study demonstrates that a combination of MZR and CsA is an effective immunosuppressant both in murine islet cell allograft survival as well as in a model of vascularized cardiac allograft. These drugs in combination suppress donor specific responses—particularly IFN-γ, IL-2 and IL-4 secreting cells along with significant suppression of various pro inflammatory cytokines and chemokines including MCP-1 and IL-6. We also demonstrate that this combination also significantly promote induction of donor specific IL-10 secreting cells and increase in serum IL-10 in mice following transplantation. Thus these synergistic actions by MZR and CsA in the immunological response to donor antigens lead to prolongation of graft survival across major histocompatibility barrier.

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Serum Neutrophil Gelatinase-Associated Lipocalin During the Early Postoperative Period Predicts the Recovery of Graft Function After Kidney Transplantation From Donors After Cardiac Death

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Purpose: Kidneys procured from donors after cardiac death hold great potential to expand the donor pool. However, they have not yet been fully used, in part due to the high incidence of delayed graft function. Although urine neutrophil gelatinase-associated lipocalin is a well-known early biomarker for renal injury after kidney transplantation, its usefulness is limited in cases with delayed graft function because of the unavailability of a urine sample. We evaluated serum neutrophil gelatinase-associated lipocalin as a potential biomarker to predict the functional recovery of kidneys transplanted from donors after cardiac death.
Materials and Methods: Consecutive patients transplanted with a kidney from a living related (39), brain dead (1) or post-cardiac death (27) donor were retrospectively enrolled in the study. Serum samples were collected serially before and after kidney transplantation. Serum neutrophil gelatinase-associated lipocalin was measured using the ARCHITECT® assay.
Results: Average serum neutrophil gelatinase-associated lipocalin was markedly high during the pretransplantation period. It decreased rapidly after transplantation. The slope of the decrease correlated well with the recovery period. By analyzing ROC curves we determined cutoffs to predict immediate, slow or delayed graft function requiring hemodialysis for longer than 1 week with high sensitivity and specificity.
Conclusions: These data suggest that serial monitoring of serum neutrophil gelatinase-associated lipocalin may allow us to predict graft recovery and the need for hemodialysis after kidney transplantation from a donor after cardiac death.

Key Words: kidney; kidney transplantation; LCN2 protein, human; delayed graft function; hemodialysis

Abbreviations and Acronyms

DCD = donor after cardiac death
DGF = delayed graft function
DGF-L = DGF longer than 7 days
ELISA = enzyme-linked immunosorbent assay
HD = hemodialysis
IF = immediate function
KTx = kidney transplantation
NGAL = neutrophil gelatinase-associated lipocalin
POD = postoperative day
SGF = slow graft function

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THE shortage of organ donors represents a major obstacle to the adequate treatment of patients with end stage renal disease. Transplantation from DCDs is becoming an alternative method to that from brain dead donors.[1] Although Reforms to the Organ Transplant Act were enacted in July 2010 in Japan, there are still few donations from brain dead donors. For more than 30 years the main source of cadaveric renal allografts has been DCDs.

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KTx using DCD grafts is still associated with a high incidence of DGF and/or failure to recover function during the early posttransplantation period. The incidence of failure to recover function is as small as 5% but most grafts undergo some degree of DGF.[2] DGF predisposes the graft to acute and chronic rejection, indicating that DGF is a crucial risk factor for short-term and long-term graft survival and recipient prognosis.[3–6] These situations prompted attempts to understand the etiology of DGF and establish highly sensitive prognostic markers for DGF in kidneys transplanted from DCDs.

Various clinical parameters were proposed to predict DGF based on preoperative risk factors.[7] However, no objective, reliable markers are currently available for the early diagnosis of DGF after KTx from a DCD. Several clinical definitions of DGF using urine output, a decreased creatinine ratios or an HD requirement were reported[5–10] but these clinical variables typically identify DGF only several days after KTx. Graft biopsy during the early period to identify acute tubular necrosis, and/or rejection or calcineurin inhibitor nephrotoxicity is the only way to make a timely diagnosis of DGF in kidney transplanted from a DCD.

In another study gene expression profiling was done using renal biopsy samples obtained 1 hour after KTx from DCDs.[11,12] Several genes that were significantly up-regulated in DCD kidneys were identified that might reflect graft performance and be potential noninvasive biomarkers. A candidate gene that may potentially be useful as a serum marker is the gene encoding serum NGAL.

There is accumulating evidence that NGAL is a sensitive marker for detecting kidney dysfunction. NGAL is one of the most remarkably up-regulated genes in the kidney after ischemia.[13,14] NGAL is also one of the genes that is rapidly induced in rat kidney isografts from brain dead donors.[11] NGAL expression is induced in proximal tubular epithelial cells during the regeneration process after kidney injury.[14,15] NGAL is a highly predictive biomarker for acute and chronic kidney injury.[16,17] Also, urine NGAL or NGAL immunostaining intensity may serve as an early biomarker for renal injury after KTx.[18–21] However, the usefulness of urine NGAL is limited in DGF cases due to the unavailability of a urine sample.

In a preliminary study we reported that monitoring serum NGAL may predict graft recovery after KTx from DCDs. In the current study we evaluated the usefulness of serial analysis of serum NGAL as a predictive biomarker for graft recovery after KTx from DCDs.

MATERIALS AND METHODS

Study Design

This investigation was approved by the institutional review boards at our centers. Written informed consent was obtained from each patient or legal guardian before enrollment. Consecutive patients who received a kidney transplanted from a living related (39) or brain dead (1) donor, or a DCD (27) were retrospectively enrolled in the study. The immunosuppressive regimen was similar in all patients, consisting of basiliximab, tacrolimus or cyclosporine with prednisone and mycophenolate mofetil.

Table 1 lists the clinical characteristics of patients treated with KTx from living related and brain dead donors, and DCDs. All DCDs from this hospital were classified as type IV in this study. The cause of donor death was cerebrovascular disease in all cases. Although 27 recipients required 1 to 30 days of HD after KTx due to DGF, the function of the single transplanted kidney from a DCD donor never recovered. There were no cases of rejection or calcineurin inhibitor nephrotoxicity, as confirmed by biopsy during DGF.

Table 1. *Patient characteristics*

	IF, No SGF	SGF	DGF	DGF-L
No. donors:	36	4	13	14
Living	34	3	2	–
Brain dead	1	–	–	–
Cardiac death	1	1	11	14
Mean age (range):				
Donor	51.8 (18–71)	59.8 (42–75)	47.2 (15–69)	55.9 (18–73)
Recipient	41.5 (8–65)	47.7 (31–63)	49.8 (41–59)	49.8 (30–63)
No. male/female	25/11	3/1	11/2	9/5
Mean mins ischemia time (range):				
Warm	4.9 (0–15)	8.1 (2–20)	10.8 (1–25)	11.0 (1–57)
Total	108.7 (47–407)	388.0 (213–844)	555.5 (162–972)	803.9 (266–1508)
Mean days post-Tx HD (range)	–	–	4.4 (1–7)	13.2 (9–21)
No. KTx:				
1	36	3	12	14
2	0	1	1	0
Mean mos HD duration (range)	37 (0–178)	56 (7–167)	152 (10–200)	200 (157–391)

Serum samples were collected before and after KTx, and stored in aliquots at −80C. Primary outcome variables were IF, SGF, DGF and DGF-L. IF was defined as no need for HD after kidney transplantation and serum creatinine 3 mg/dl or greater on POD 5. SGF was defined as no need for HD after kidney transplantation and serum creatinine greater than 3 mg/dl on POD 5, according to Humar et al.[22] DGF was defined as the need for HD within the first few weeks after transplantation.[23] DGF-L was defined as the need for HD longer than 7 days in duration. The decision to initiate HD was made by the primary transplant nephrologists and transplant surgeons without our involvement. Other variables included age, gender, original kidney disease, warm and total ischemia time, urine output and serial serum creatinine.

ELISA for NGAL Quantification

ELISA for serum NGAL was done as previously described.[16] Briefly, microtiter plates were precoated with mouse monoclonal antibody raised against human NGAL (HYB211-05, AntibodyShop, Gentofte, Denmark) and blocked with buffer containing 1% bovine serum albumin. The wells were then coated with 100 μl of serum samples or standards (NGAL 1 to 1,000 ng/ml) and incubated with biotinylated monoclonal antibody against human NGAL (HYB211-01B, AntibodyShop), followed by avidin-conjugated horseradish peroxidase (Dako, Carpinteria, California). TMB substrate (BD™ Biosciences) was added for color development, which was read after 30 minutes at 450 nm with a Benchmark™ Plus microplate reader. All measurements were made in triplicate. The interassay and intra-assay coefficient of variation was 5% to 10% for batched samples analyzed on the same day. Laboratory investigators were blinded to sample sources and clinical outcomes until the end of the study.

ARCHITECT NGAL Assay

The ARCHITECT NGAL assay uses a noncompetitive sandwich format with chemiluminescent signal detection, as described previously.[24] The assay includes a microparticle reagent prepared by covalently attaching anti-NGAL antibody to paramagnetic particles and a conjugate reagent prepared by labeling a second anti-NGAL antibody with acridinium. Mouse anti-NGAL antibodies were developed elsewhere to be directed against distinct, nonoverlapping NGAL epitopes. Calibrators were prepared with recombinant human NGAL expressed and purified elsewhere. The recombinant NGAL was a full length protein.

The assay uses an automated sequence consisting of an 18-minute incubation step with the sample (2.5 μl) and microparticle reagent (50 μl), a solid phase wash step and a 4-minute incubation step with conjugate reagent (50 μl). After the immunochemistry steps the solid phase is washed again and the acridinium label is triggered with peroxide and base to generate the signal. Assay calibrators are at 0, 10, 100, 500, 1,000 and 1,500 ng/ml. The measured NGAL concentration is proportional to the signal.

Statistical Analysis

Statistical analysis was done using PSAW Statistics, version 18 (SPSS®). Results are shown as the mean ± SEM. Correlations were evaluated by linear straight line regression. ROC analysis was performed to assess the potential of serum NGAL to predict DGF. AUC was calculated from a standard ROC plot. The optimal cutoff was defined by the largest sum of sensitivity and specificity. Significance was considered at $p < 0.05$.

RESULTS

Since the ARCHITECT NGAL assay was optimized to estimate urinary NGAL, we first measured serum levels using that assay and compared the data with those obtained by standard NGAL ELISA. The cross-sectional pilot study was designed to verify the ARCHITECT NGAL assay against the NGAL ELISA assay. The NGAL concentrations in the 512 serum samples from this study determined by the 2 assay correlated highly ($r = 0.95$, fig. 1). Using the ELISA assay the average serum NGAL of the 173 pre-HD and post-HD chronic renal failure cases was 945 ± 27 and 762 ± 33 ng/ml, respectively. In contrast, using the ARCHITECT NGAL assay the average serum NGAL of the 173 pre-HD and post-HD cases was 735 ± 22 and 578 ± 24 ng/ml, respectively.

The study included 67 renal transplant recipients, including 36 with IF, 4 with SGF, 13 with DGF and 14 with DGF-L (table 1). In KTx cases with IF serum NGAL decreased rapidly from POD 1 to PODs 2, 3 and 5 (mean 229 ± 15, 170 ± 13, 159 ± 10 and 126 ± 9 ng/ml, respectively, table 2). In contrast, in KTx cases with SGF serum NGAL decreased relatively slowly from POD 1 to PODs 2, 3 and 5 (mean 371 ± 67, 254 ± 75, 251 ± 52 and 205 ± 22 ng/ml, respectively). However, in recipients of a kidney transplant from DGFs in whom HD was required for less than 1 week (DGF 7 days or less) serum NGAL decreased gradually from POD 1 to PODs 2, 3 and 5 (mean 757 ± 58, 560 ± 60, 508 ± 60 and 355 ± 48 ng/ml, respectively). In DGF cases in

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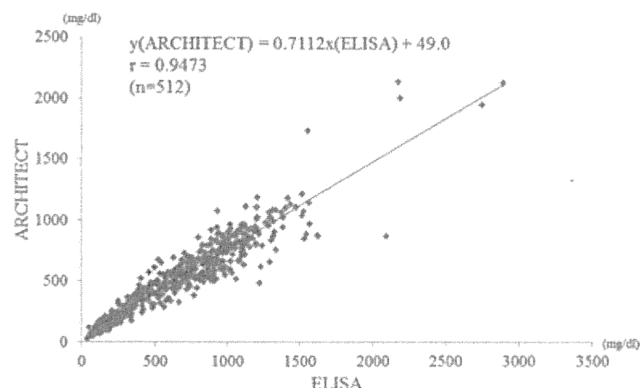

Figure 1. Straight line linear regression analysis shows correlation of values obtained by ARCHITECT NGAL assay vs standard ELISA.

Table 2. *Serum NGAL time course in patient groups*

	Mean ± SEM NGAL (ng/ml)				
	Pre-KTx	POD 1	POD 2	POD 3	POD5
IF	684 ± 63	229 ± 15	170 ± 13	159 ± 10	126 ± 9
SGF	853 ± 13	371 ± 67	254 ± 75	251 ± 52	205 ± 22
DGF	670 ± 39	757 ± 58	560 ± 60	508 ± 60	355 ± 48
DGF-L	762 ± 72	849 ± 56	678 ± 68	666 ± 46	588 ± 36

which HD was required for greater than 1 week, serum NGAL decreased more slowly from POD 1 to PODs 2, 3 and 5 (mean 849 ± 56, 678 ± 68, 666 ± 46 and 588 ± 36 ng/ml, respectively). The slope of the decrease correlated well with the period needed for recovery (fig. 2).

To further evaluate NGAL as an indicator of renal function recovery we analyzed the correlation between NGAL on early PODs and various clinical parameters on subsequent PODs by linear regression analysis. As expected, DGF duration correlated well with NGAL levels on POD 1 (fig. 3, *A*).

Regarding clinical parameters, we evaluated urine output, serum potassium and serum creatinine on POD 5. Urine output and serum creatinine on POD 5 correlated well with NGAL levels on POD 1 (fig. 3, *B* and *C*). We also evaluated the NGAL decrease from POD 0 to 2, which only weakly correlated with DGF duration (fig. 3, *D*).

To assess the usefulness of serum NGAL measurements at varying cutoffs to predict the clinical outcome of IF, SGF, DGF and DGF-L, conventional ROC curves were generated and the AUC was calculated on PODs 1 to 3 and 5. To predict graft failure, including SGF, DGF and DGF-L, we applied

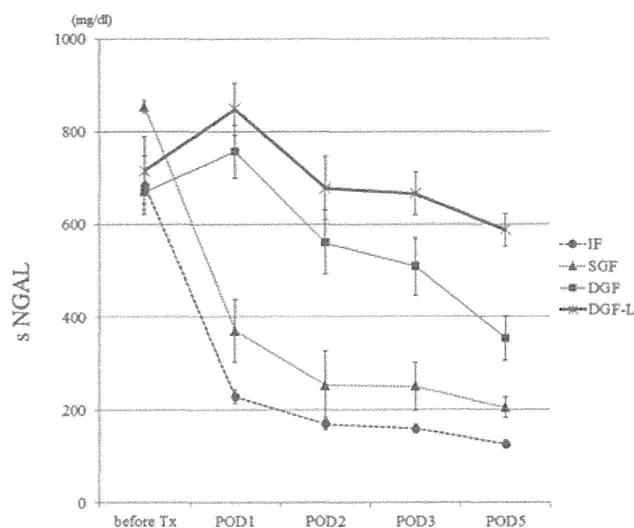

Figure 2. Mean serum NGAL measured by ARCHITECT assay in early post-KTx period. Vertical lines indicate SE. *Tx*, KTx.

a cutoff of 350 ng/ml at POD 1. This allowed for high sensitivity (88%) and specificity (96%), and suggested that serum NGAL was diagnostic for predicting graft failure (ROC AUC 0.98, table 3). To predict the need for HD (DGF plus DGF-L) we applied a cutoff of 500 ng/ml at POD 1. This allowed high sensitivity (91%) and specificity (97%) (AUC 0.99, table 3). To predict DGF-L we applied a cutoff of 700 ng/ml at POD 1. This also allowed high sensitivity (82%) and specificity (83%) (AUC 0.93, table 3).

Using a similar approach serum NGAL at POD 2 was also an excellent biomarker for predicting DGF at a cutoff of 350 ng/ml with 86% sensitivity and 90% specificity (AUC 0.94). DGF-L at a cutoff of 600 ng/ml had 73% sensitivity and 85% specificity (AUC 0.85). POD 3 serum NGAL was also an excellent biomarker for predicting DGF at a cutoff of 300 ng/ml with 91% sensitivity and 93% specificity (AUC 0.98) while DGF-L at a cutoff of 500 ng/ml had 82% sensitivity and 85% specificity (AUC 0.94).

DISCUSSION

We evaluated the usefulness of serum NGAL as a predictor of graft function recovery after KTx. This relationship is not well established since the kinetics of serum NGAL during the peri-KTx period are complicated. Various factors can affect serum NGAL, including the condition of the native kidneys, the pre-KTx condition, and post-KTx functional recovery of the graft and clearance via HD or urine. In our previous study the pattern of the serum NGAL decrease appeared to be biphasic after transplanting kidneys from DCDs within the initial few weeks after KTx and the multifactorial nature hindered its application as the biomarker during this period.[25] It was also reported that even donor serum NGAL values obtained before KTx were not useful to predict graft function recovery.[26] Thus, we analyzed serum NGAL during the early period after KTx as a biomarker to predict graft function recovery. Although values before KTx varied widely according to the clinical condition of each case, those on POD 1 correlated well with the duration of subsequent kidney dysfunction.

POD 1 levels were useful to predict the prognosis regardless of serum NGAL before KTx in each case.