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.

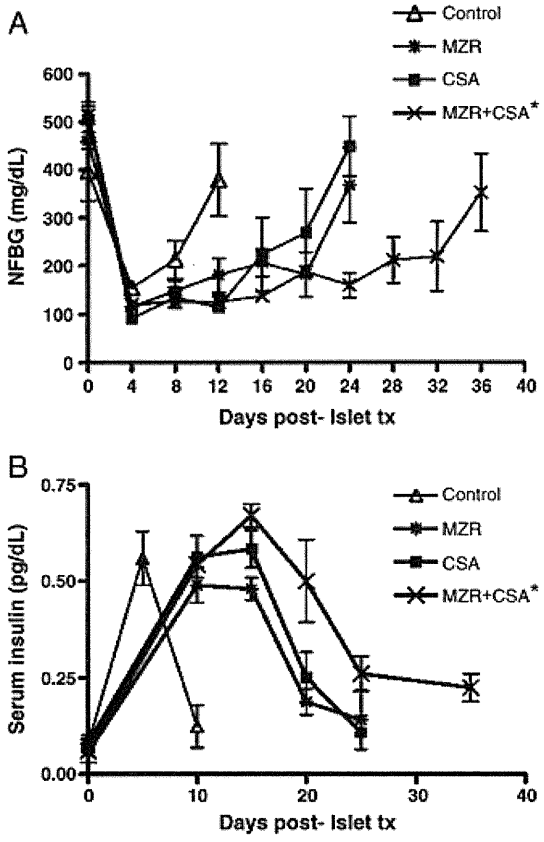

Fig. 1. Islet allograft survival and function among the recipients treated with mizoribine (MZR), cyclosporine (CsA) or a combination of both. Data is representative of mean ± SE of 10 animals/group at each time point of blood draw. *indicates $p<0.05$ in comparison to other groups. (1A) Non-fasting 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

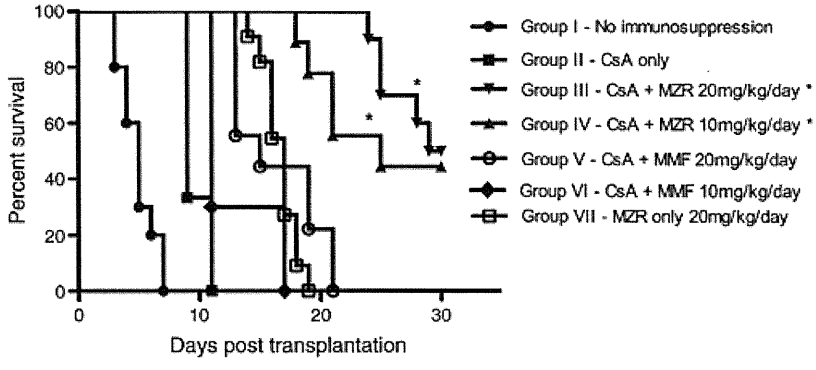

Fig. 2. Kaplan–Mier survival curve of Murine heterotopic heart allograft survival among the groups of different immunosuppressive regimens. Groups with CsA and MZR (Group III and IV) and significantly higher allograft survival (log rank $p=0.001$). Each group consisted of 10 animals each. *indicates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

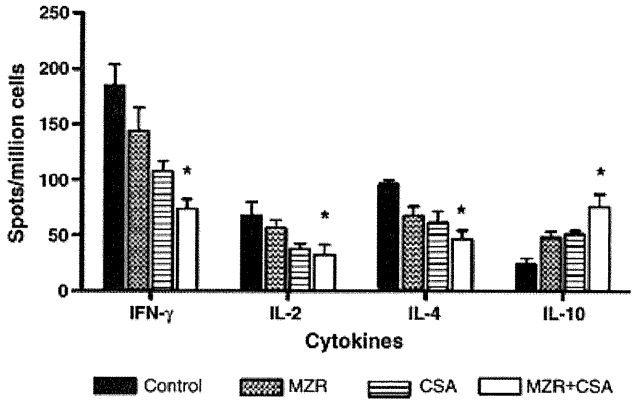

Fig. 3. ELISpot to enumerate IFN-γ, IL-10, IL-4, IL-2 secreting cells in response to irradiated donor splenocytes. Recipients treated with CsA and MZR demonstrated significantly decrease IFN-γ, IL-2, IL-4 secreting cells with increased IL-10 secreting cells in response to donor cells. Control group did 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

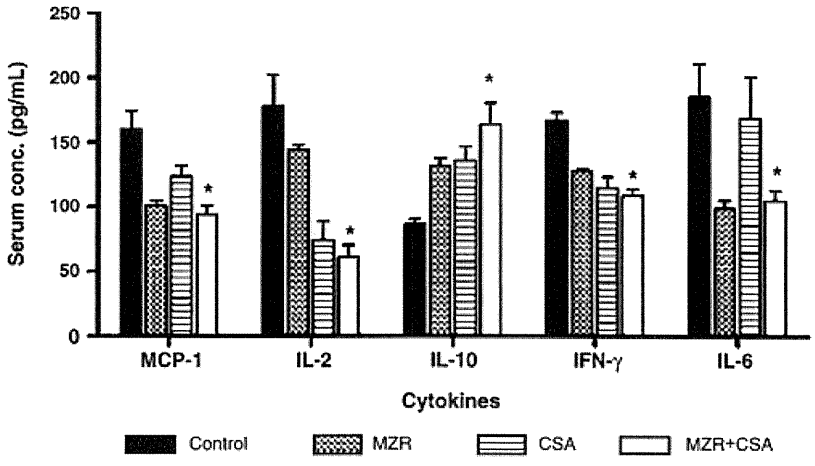

Fig. 4. Serum cytokine and chemokine concentration measured by Luminex. Recipients treated with CsA and MZR had decreased serum IFN-γ, IL-2, IL4, IL-6 and MCP-1 and increased IL-10 compared to other groups ($p<0.05$). Control group did not receive any immunosuppression (data expressed in pg/mL ± SE). 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.

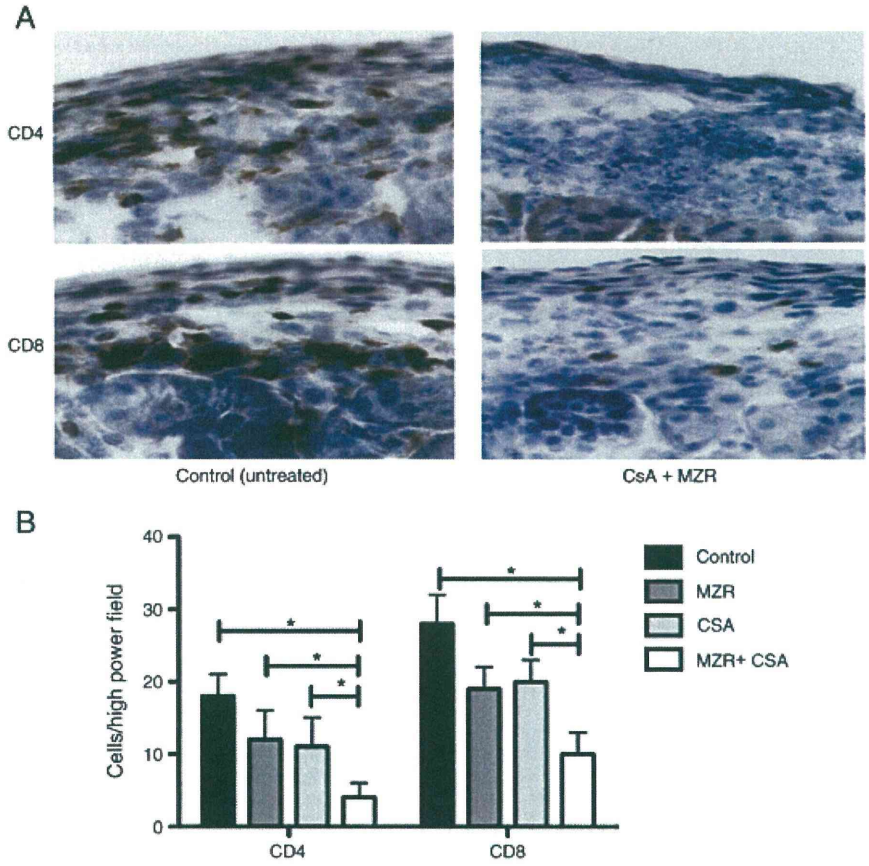

Fig. 5. Immunohistological analysis of islet cell grafts treated with different immunosuppressive drugs. Kidney from recipients of islet allo-transplantation was harvested on day 7 post-transplant. CD4 and CD8 cells were stained and number of cells/high power field were counted in 3 areas of 3 sections 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. T1

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. F1

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 T2

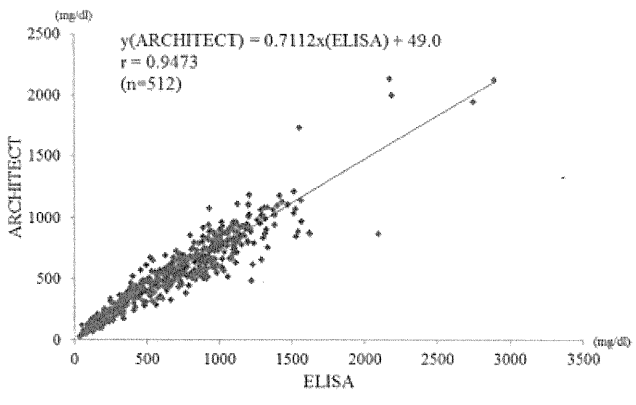

Figure 1. Straight line linear regression analysis shows correlation of values obtained by ARCHITECT NGAL assay vs standard ELISA.

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 F2 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 F3 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 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 T3 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).

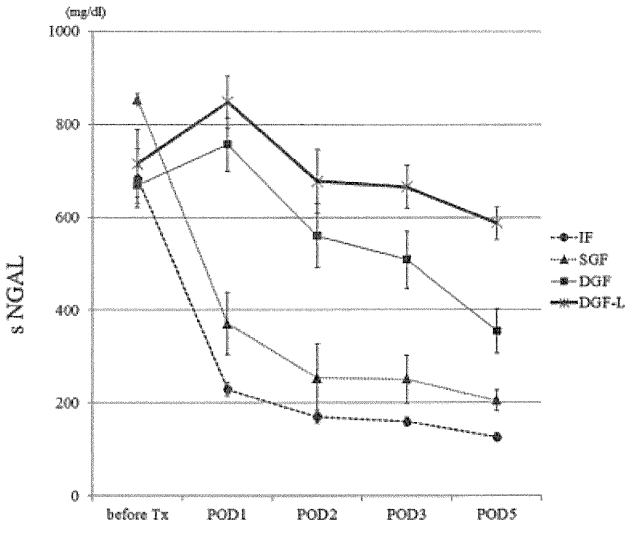

Figure 2. Mean serum NGAL measured by ARCHITECT assay in early post-KTx period. Vertical lines indicate SE. *Tx*, KTx.

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.

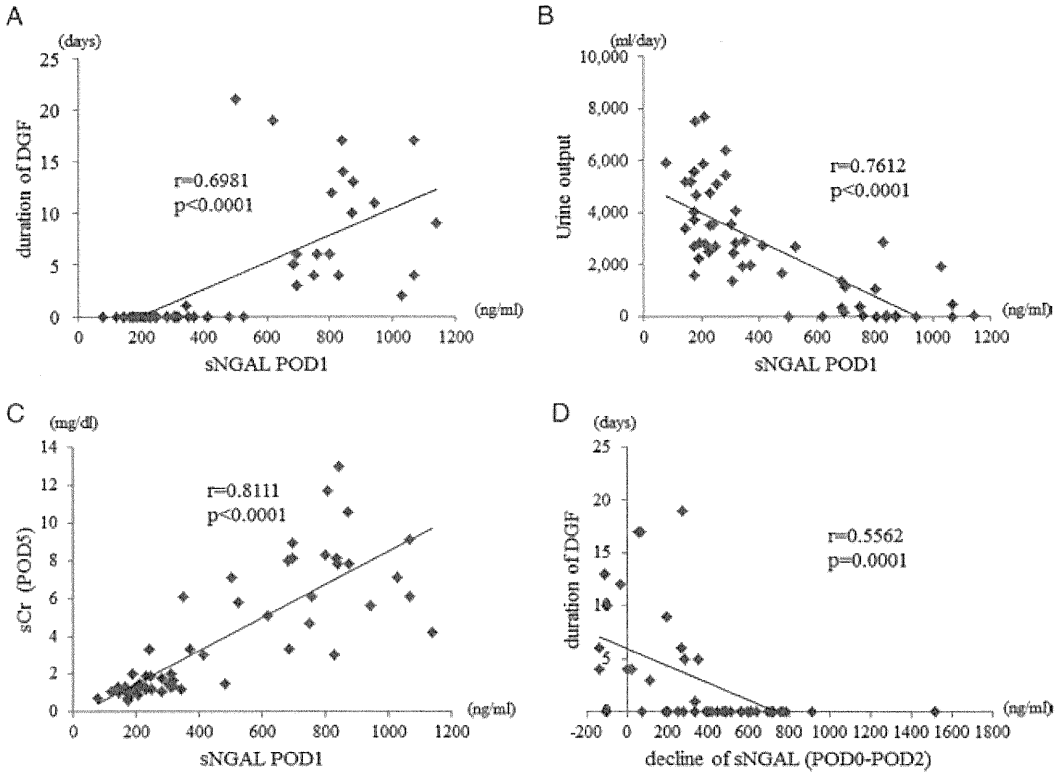

Figure 3. Serum NGAL (*sNGAL*) correlation with clinical parameters. *A*, POD 1 NGAL and DGF duration. *B*, POD 1 NGAL and POD 5 urine output. *C*, POD 1 NGAL and POD 5 serum creatinine (*sCr*). *D*, NGAL decrease from POD 0 to 2 and DGF duration.

The 2 major factors determining how serum NGAL decreased were 1) the decrease in production from the graft due to recovery from acute kidney injury during the transplantation procedure and 2) the increase in urine volume, also due to the recovery of graft function. However, serum NGAL began to decrease even before urine volume recovered, as observed in our previous study.[25] This finding suggests

Table 3. *Derived sensitivity and specificity after KTx at various serum NGAL cutoffs*

	NGAL Cutoff (ng/ml)								
POD	200	250	300	350	400	450	500	600	700
1:									
Group				SGF			DGF		DGF-L
AUC				0.98			0.99		0.93
Sensitivity				0.88			0.91		0.82
Specificity				0.96			0.97		0.83
2:									
Group			SGF	DGF				DGF-L	
AUC			0.90	0.94				0.85	
Sensitivity			0.80	0.86				0.73	
Specificity			0.89	0.90				0.85	
3:									
Group		SGF	DGF				DGF-L		
AUC		0.96	0.98				0.94		
Sensitivity		0.84	0.91				0.82		
Specificity		0.93	0.93				0.85		
5:									
Group	SGF	DGF			DGF-L				
AUC	0.96	0.97			0.98				
Sensitivity	0.84	0.91			0.91				
Specificity	0.93	0.97			0.89				

that the decrease in serum NGAL reflects a decrease in production by the injured kidney graft.

It is still unclear why increased circulating NGAL during the early post-KTx period is mostly derived from de novo production of the injured kidney graft and not from preexisting circulating NGAL. NGAL is one of the lipocalin species, which includes rapid turnover proteins such as retinol binding protein.[27] Serum NGAL is considered a sensitive marker for acute kidney injury or infection due to its detection sensitivity as well as its rapid decrease in the recovery period.[28] However, this decrease in serum NGAL is supposedly due to rapid excretion from urine. It cannot explain the rapid decrease observed before urine volume recovers.

The change in blood flow from native kidney to graft may at least partly affect the origin of circulating NGAL on POD 1. Regardless of the mechanism, serum NGAL on POD 1 mainly originates from de novo production by the graft and reflects graft injury, predicting future graft function.

Our current data indicate that the serum NGAL level on POD 1 is a highly sensitive and specific marker predicting future graft function. The usefulness of urine NGAL as a noninvasive marker for this purpose is well established.[19,,20,29] However, prognostic biomarkers are required that can be used during the early post-KTx period, specifically in the anuric period of DGF. This is particularly true when kidneys are transplanted from DCDs since DCDs are the primary source of the renal grafts used in Japan.

NGAL measurement was previously reported to have an advantage over serum creatinine measurement to predict acute renal injury due to its rapid response to changes in kidney function.[16] Serum NGAL has limited usefulness due to multiple confounding factors. However, serum samples, which can be obtained noninvasively from patients who require HD during the anuric period, may be an alternative source of diagnostic samples. Furthermore, using the ARCHITECT assay for serum samples would also facilitate serum NGAL measurement as a rapid diagnostic test in the clinical setting.

On POD 1 when we obtain information about the prognosis for the transplanted kidney graft by measuring serum NGAL, multiple options can be chosen based on the values. A potential clinical use of such early prediction of graft function is determining the optimal timing for weaning off HD. This may be achieved without the need for invasive tests such as kidney biopsy, which are currently the most reliable diagnostic method. In patients with high serum NGAL clinicians may have the option of decreasing the dose of immunosuppressant, such as cyclosporin A or tacrolimus, to circumvent potential kidney injury. Furthermore, our findings will promote research to elucidate the etiology of graft failure as well as the development of effective methods to prevent dysfunction. In conclusion, although multiple factors may potentially affect values, our findings indicate that serum NGAL on POD 1 is a sensitive, specific and noninvasive biomarker to predict graft recovery after KTx.

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特集「法改正後の移植の現状と問題点：腎膵領域」

脳死下献腎移植と心停止下献腎移植の現状と問題点

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はじめに

改正臓器移植法が2010年7月17日から施行され，1年6カ月が経過した。脳死下での臓器提供が増加しつつあるものの，献腎移植に関しては，献腎移植希望登録者1万2千人に対し，献腎提供は，従来行われてきた心停止下献腎提供と併せても年間110件前後にとどまり，平均待機年数は約15年と長く，臓器提供に関するいっそうの普及啓発が必要である。

普及啓発の変化

改正臓器移植法が施行され，1年半が既に経過した。施行当初，マスコミにも大きく取り上げられ，広く法改正の内容が周知されることとなった。内閣府が行った臓器移植に関する世論調査は，残念ながら2008年以後行われていないため[1]，法改正後の国民の意識変化を客観的に知るすべはない。しかし，2010年8月10日の法改正後の1例目から2011年4月13日の本邦初の法改正後15歳未満のドナーからの脳死下臓器提供等に至る内容は，新聞テレビ等を中心とするマスコミで全国に報道された。また，地域や県単位での初の法改正後脳死下臓器提供の場合，法改正当初の全国的な報道には至らないものの，記者会見が行われ，地域での報道が行われている。これにより，臓器移植あるいは法改正の内容が少しずつ広く周知され，徐々にではあるが国民の意識にも変化がみられると推測される。実際，心停止下での腎提供を多く行ってきた当院でも，法改正前には1例のみであった脳死下臓器提供が，法改正後3例の脳死下臓器提供，3例の脳死下腎移植が行われた（図1）。また，心停止下の腎提供の場合においても，近年ドナーファミリー側からの申し出が増加しつつあり，さらに院内移植コーディネーターからドナーファミリーへの説明の場においても，既に臓器提供，臓器移植に関する国民の認識が少しずつ変化していることがうかがわれ，啓発活動が徐々にではあるが進みつつある。

献腎提供の実際

法改正後脳死下での臓器提供数，移植数は著明に増加した。日本臓器移植ネットワークの報告によると[2]，法改正2010年7月17日からの1年間での脳死下臓器提供は55件，2011年度1年間で44件の脳死下臓器提供が行われた。未曾有の被害をもたらした東日本大震災の発生の影響もあってか，2011年3月は脳死下臓器提供が行われなかったものの，それを除けば，全国で月4件前後のペースで脳死下臓器提供が行われていたこととなる（図2，3）。

臓器提供数の推移は，2009年は脳死下7件に対し，心停止後98件の合計115件であったのが，2010年には脳死下32件に対し，心停止後81件の合計113件，2011年は脳死下44件に対し，心停止後68件の合計112件と，合計ではこの3年間で変化していない（図2）。言い換えると，従来は心停止下での臓器提供であった症例が，法改正に伴い脳死下臓器提供に移行したため，臓器提供総数では変化していないと考えられる。

また，腎移植に限ってみると，この3年間で2009年は献腎移植数182件（そのうち，脳死下7件），2010

図1　1997~2011年における当院の心停止下献腎提供ならびに脳死下臓器提供と心停止下献腎移植数

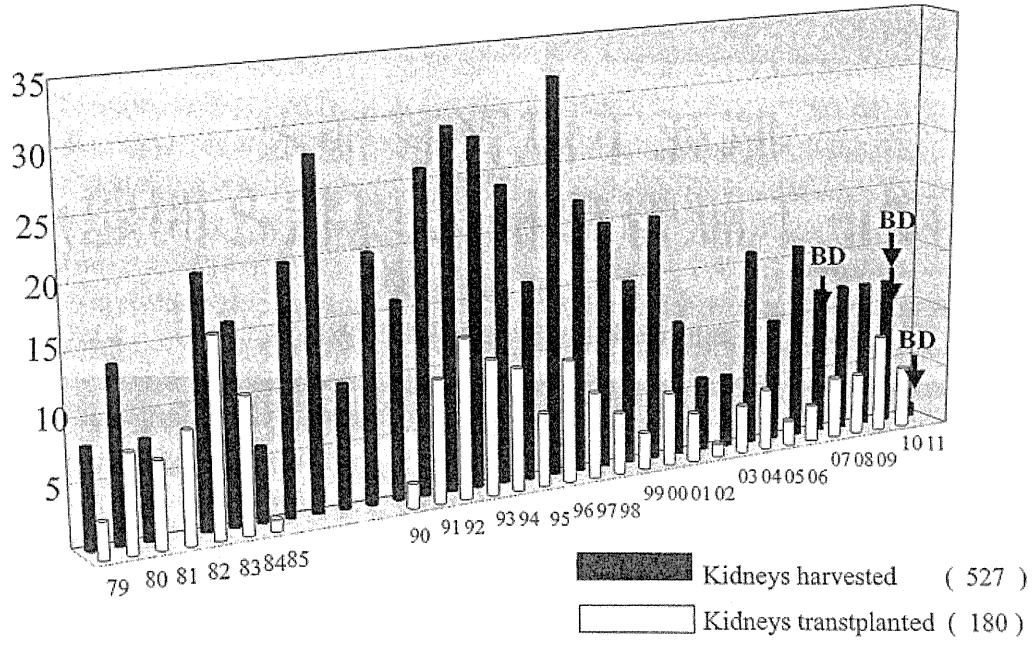
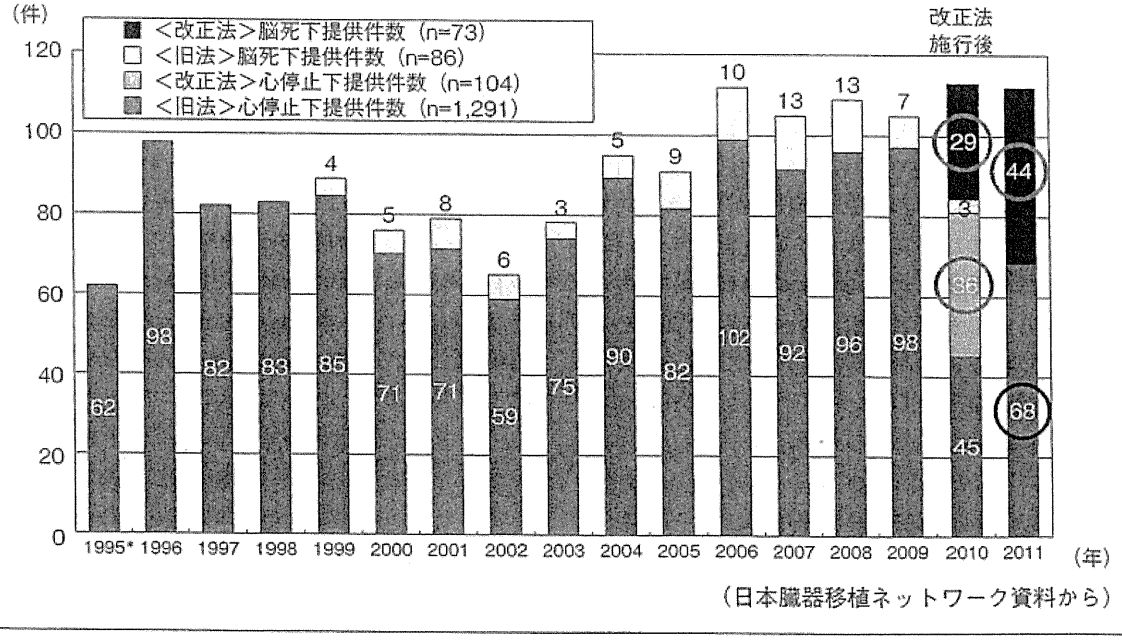
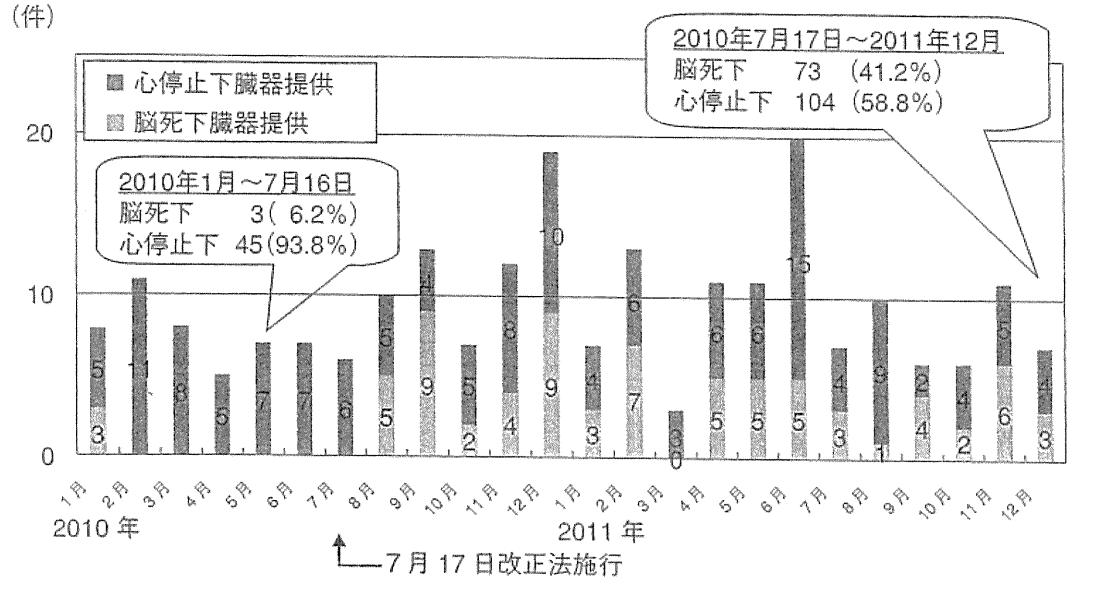


図2　臓器提供件数の年次推移



(日本臓器移植ネットワーク資料から)

図3　2010~2011年における臓器提供件数の月別推移



（日本臓器移植ネットワーク資料から）

年は186件（そのうち，脳死下39件），2011年は182件（そのうち，脳死下57件）であり，脳死下での腎移植数が増えたのみで，臓器提供数同様，総数は変化していない。一方で膵腎同時移植を例にとると，2009年は7件に対し，2010年は23件，2011年は29件と明らかな増加が認められる。

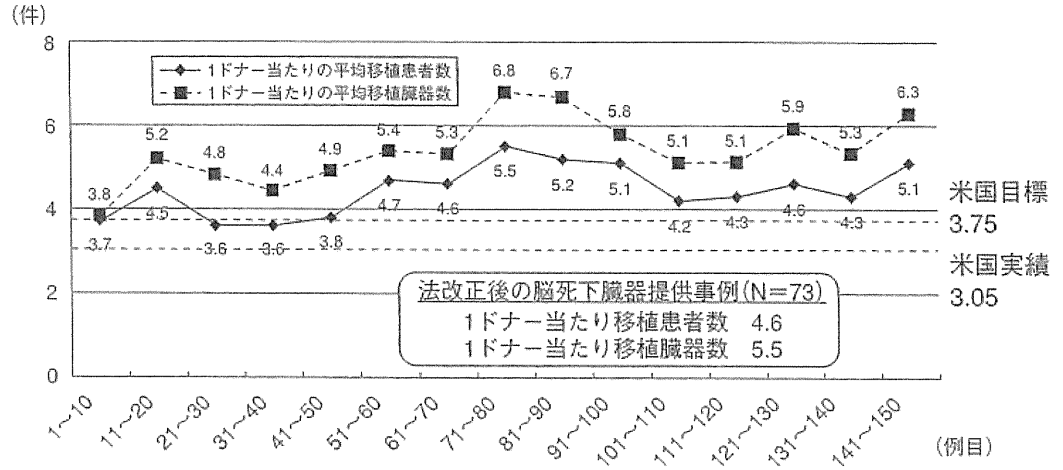
移植希望登録患者は2012年1月5日現在で，腎移植希望者12,509人，このうち肝腎移植希望者は10人，膵腎希望者は152人である。平均待機期間は腎移植希望者が約15年に対し，膵腎移植希望者は約3年であり，大きな隔たりをみせている。2011年10月末での脳死下腎移植ならびに脳死下膵腎移植の待機日数は，腎が平均5,483日（125～9,853日，最短は16歳未満症例），膵腎は平均1,383日（53～4,135日）であり，希望者数が1/80である膵腎移植希望者の待機日数が腎移植希望者の1/4の待機期間で移植を受けていることとなる（いずれのデータも日本臓器移植ネットワーク資料）。

法改正に伴い，臓器提供数の飛躍的な増加が期待されたが，献腎移植に限っては，待機期間の短縮を含め，法改正前とまったく状況が変化していないのが実情である。

ドナー管理と臓器評価に関する変化

従来法の下では，ほとんどの症例で心停止下の腎単独採取，あるいは一部で膵島分離の目的で腎採取後に膵摘出が行われていた。骨や眼球等の提供は，腎採取の後，場合によっては摘出チーム到着が遅れる場合，腎採取とは独立して行われていたが，法改正とともに，脳死下多臓器提供が増加した（図3，4）。このため，臓器採取に関する事情も変化した。心停止下では，日本臓器ネットワークからの連絡により腎摘出チームが派遣され，提供病院施設内で待機することとなる。ドナー管理に関しては，尿量，血圧維持に関する昇圧剤の変更等を中心に協力することとなるが，あくまで腎摘出チームが直接ドナー管理を行うことはない。状況に応じて，ドナー担当医とドナー家族の関係に十分配慮する必要があり，積極的にドナー管理に介入できないことも多い。一方脳死下臓器提供の場合は異なる。第1回目脳死判定以降に提供病院にメディカルコンサルタント（MC）が派遣され，第1回目脳死判定以降からドナー管理を行うこととなる[3]。基本的に呼吸循環管理を中心に行われる。MCの導入により積極的な呼吸循環管理が行われ，臓器提供数も増加した（図4）。積極的な気管支鏡の管理が行われ，肺移植後の生存率も改善されている[3]。腎に関しても，MCによる積極的な呼吸循環管理を中心とするドナー管理

図4　1ドナー当たりの移植患者数・移植臓器数と米国の比較



移植臓器数：心肺同時移植は3臓器，両側片肺移植および膵腎同時移植は2臓器，分割肝移植は1臓器と数える。
※米国では，2006年の1ドナー当たりの平均移植臓器数が3.05であり，3.75を到達目標に設定し増加に取り組んでいる。

（日本臓器移植ネットワーク資料から）

は，腎および腎機能を良好に維持していることが推測される。ドナー腎の評価については，心停止下の場合派遣された摘出チームが行うこととなる。一方，脳死下では，第一次評価として日本臓器移植ネットワーク（JOT）コーディネーター（Co）が提供病院に赴き，禁忌事項の確認を行う。第二次評価は第1回目脳死判定以降MCが臓器ごとに評価を行う。そして，第三次評価は実際に移植を行う移植施設のスタッフが行うこととなる。心停止下と脳死下での腎採取では，心停止下の場合は摘出チーム（必ずしも腎移植施設とは限らない）がドナー評価を行い，脳死下の場合は摘出チームが移植施設であり，移植施設自らが評価を行うことが相違である。

献腎採取術に関する変化

腎採取に関しては，心停止下の腎提供の場合，脳死下に家族の同意が得られている場合には，心停止前にダブルバルーンカテーテルを用いたカニュレーションを行う。カテーテルの挿入は，脳死判定が終了している場合には文書による家族の承諾を得た上で，血圧が50 mmHg程度まで下降した時期に，脳死判定ができていない場合には心停止直後に行われる[4]。一方，脳死下での腎提供の場合，多臓器提供の中での腎採取となり，ドナーごとに提供が行われる臓器数と対象臓器が異なる。このため，全国から集まった摘出チームの間でミーティングが行われ，採取術前のディスカッションが重要となる。

言うまでもなく，心停止下の場合腎採取を行う時間を予定することはできない。ドナーの状態により，摘出チームは長期間の待機を提供病院で要する場合もある。低血圧での死線期の遷延や，ドナー担当医からドナー家族への死亡宣告，グリーフケアに要する時間等，報告書のみでは把握できない腎虚血時間が実際には存在することとなる。本邦では，記録上温阻血時間（WIT）は死亡宣告から灌流開始時時間である。ダブルバルーンカテーテルを用いた体内局所灌流冷却は，虚血時間の短縮につながり，採取するグラフトの質低下を予防する。一方，脳死下の場合，臓器採取は予定を組んで，そのタイムテーブルに沿って行われることとなる。このため，摘出チーム出発から，実際の臓器摘出に至ってはある程度時間予測が可能となる。カニュレーションはドナー心拍動下に腹部大動脈に腹腔内の多臓器採取術中に行われる。下大静脈から脱血用カテーテルを挿入し，横隔膜の位置でクロスクランプの後，ただちに灌流が行われ，冷却も腹腔内臓器全体で行われることとなる。記録上もWITは存在せず，心停止下と比較すると格段に条件の良い状態で腎採取が行われることとなる。当院における脳死ドナーからの献腎移植症例では，3例ともすべてimmediate function

であり，従来の心停止ドナーからの献腎移植と比較し，術後経過は生体腎移植に近い経過をたどっている。腎保存液としては脳死下の場合，腹腔内臓器は他臓器とともに一律UW液で灌流される。一方，心停止下の場合は摘出チームによって選択されるため，灌流液，保存液を含めてEuro-Collins液やUW液などさまざまである。現時点で保存液に関しては腎に関して限定する必要はないと考えられるが，保存効果の向上のためにはUW液を使用すべきと考える。一方，灌流保存液については，UW液は保存期間や費用などの問題がある。多方面で新規臓器灌流保存液の開発が現在進められており，今後臨床応用が検討される必要がある。

腎採取術については，基本的に心停止下，脳死下ともに大きな変化はない。従来心停止下腎採取術のみを行っていた摘出チームは，脳死下においては多臓器採取のチームと共同協調して手術に参加することとなる。臓器採取は予定通りに進められるため，虚血時間の影響を考慮し他臓器チームの操作が優先され，腎採取の操作は他チームの後となる。膵腎の提供と同時の場合は，膵腎一塊として採取の後，膵腎のチームから対側腎を分離してもらうため，採取術で行う操作は単独としてほとんど存在しない。しかし，術前のドナー評価により，法改正後脳死下でも腎単独採取となったケースが存在する。この場合，従来心停止下腎採取のみ行っていたチームにとっては，腎採取そのものに関する操作は心停止下と同様であるが，心拍動下に腹部大動脈にカニュレーションを行い，クロスクランプをかける操作など，慣れない状況での対応が必要となる。しかし，摘出される腎は，従来の心停止下臓器提供における死線期の影響を受けることはなく，慎重に操作を行えば，心停止下以上に良好な成績を残すと考えられる。

腎採取後の臓器搬送その他

他臓器と違い，腎単独の場合は心停止下あるいは脳死下でも同一県下や同一ブロック内での移植となる。搬送と移動に関しては，他臓器におけるような全国規模の移動を要することはない。臓器搬送については心停止下の場合，日本臓器移植ネットワークが腎を搬送するが，脳死下の場合は摘出チームが自身の移植施設に持ち帰ることとなる。今後，他臓器に関しては，脳死下臓器提供の数が増加すれば，現在のように遠方へ摘出に移動し，臓器を持ち帰ることが見直される可能性がある。一方腎の場合は，同一県下や同一ブロック内と，移動範囲も比較的限定されており，従来の方法で変更の必要はないと推測される。

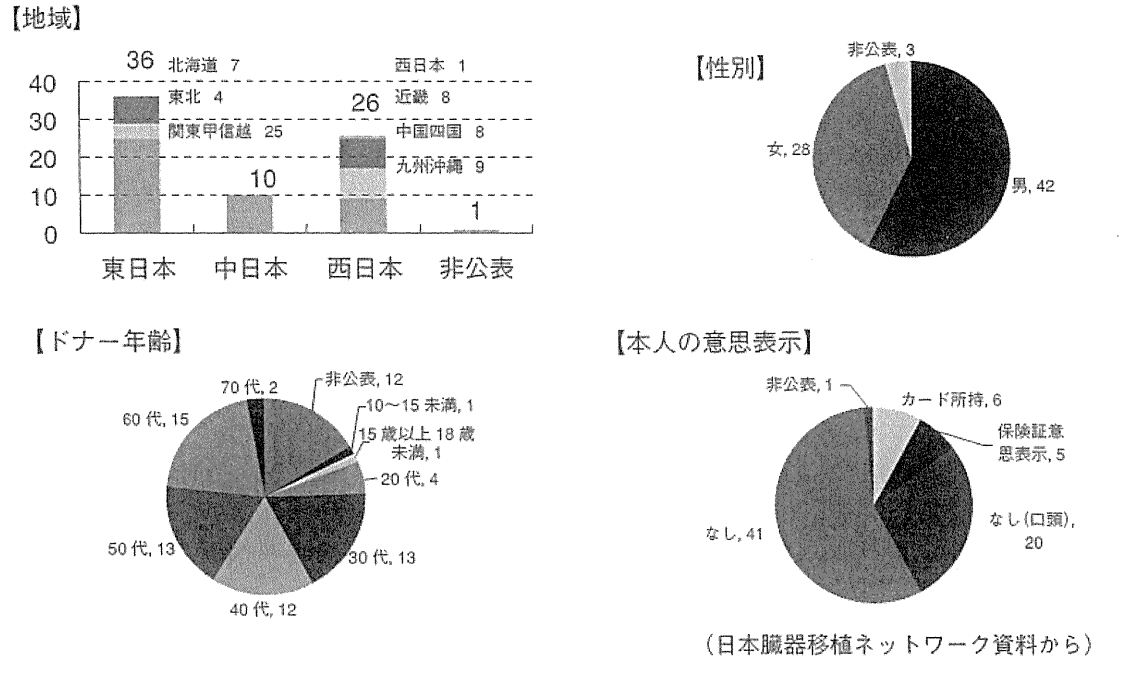
実際に法改正後2011年10月末までに行われた脳死下献腎移植と心停止下献腎移植において，総阻血時間（TIT）は脳死下献腎移植が平均8時間2分（2時間49分～19時間16分），心停止下献腎移植が平均12時間50分（3時間2分～34時間57分）と明らかに脳死下においてTITが2/3と短い（いずれのデータも日本臓器移植ネットワーク資料）。理由については諸事情を考慮する必要があるが，心停止下腎提供においては突然のドナー心停止に伴うレシピエント選定の遅れや，レシピエント候補者への連絡に対し，今回の移植は見送りたいなどの理由で，候補者が次々に辞退され下位にまわり，レシピエント決定まで長時間を要する場合が推測される。一方，脳死下の場合は腎採取が予定時間通り行われることが多く，レシピエント選定においても脳死下臓器提供の普及啓発により受け入れがよく，結果としてTITの短縮に至ると考えられる。

本邦の献腎移植成績と今後の展望

本邦の献腎移植成績は年代別の生存率，生着率については，年代ごとでの改善がみられ，1982年以前は，5年生存率54.0%，5年生着率35.6%であったものが，2001年以降では，5年生存率89.1%，5年生着率77.8%と飛躍的に改善している[5]。この成績は，米国の脳死下臓器提供を中心とする2002～2007年の献腎移植成績（expanded criteria donors：ECD；5年生存率72%，5年生着率57%，non-ECD 5年生存率84%，5年生着率72%）と比較しても[6]，本邦の心停止下献腎移植の多くがECDであることを考慮すると，本邦の献腎移植は世界に誇る移植成績と考えられる。

法改正後の臓器提供数の推移を踏まえて，献腎移植数を増加させるには，言うまでもなく本邦の臓器提供全体を増加させる必要がある。普及啓発として脳死下での臓器提供に対して理解が進む一方，心停止下での腎提供も従来通り可能であることは今後も継続して啓発すべきである。法改正後の脳死下臓器提供においてもドナーカード所持は73例中6例，保険証意思表示5例にとどまり，本人の意思表示による臓器提供は依然少ないのが現状である（図5）。臓器提供に際し，本人の意思表示が明確であれば，臓器提供に至りやす

図5　法改正施行後の脳死下臓器提供の内訳（n=73，2011年12月末時点）



（日本臓器移植ネットワーク資料から）

いと考えられ，一方意思表示が明確でない場合は，多くをドナー家族の同意により臓器提供を受けることとなり，ドナー家族への心理的負担は肉親の危機的状況下で臓器提供という決断を強いるため，提供後はグリーフケアを含めた対応が重要と考えられる。今後臓器提供を増加させるためには，広く国民の理解を求め，普及啓発活動においても地道に繰り返して行うことが重要と考えられる。臓器提供特に献腎移植の飛躍的な増加を期待してやまない。

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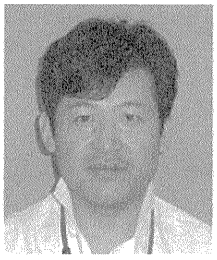
改正臓器移植法後の臓器提供に対する医療機関の責任
――臓器提供を適正かつ安全に行うためのシステム構築

Responsibility of the medical institution for the organ donation after revised act on organ transplantation



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◎2010年の改正臓器移植法施行後，脳死下臓器提供症例は増えつつある．増加の大きな理由としてあげられているのは，提供臓器の摘出が家族の意思でも可能となったことである．これまでも心停止下臓器提供(腎，角膜，膵)は家族の意思があれば可能であったが，今後は本人や家族の意思によっては，より多くの脳死下臓器提供対応が求められる．つまり改正臓器移植法は個々のさまざまな価値観を通じ，各医療機関の対応を迫ることになったともいえる．そして価値観や意思が多様化していくなかで，提供病院における体制整備についての取り組むべき課題も増加した．つまり重要なことは，医療現場を支える院内体制の整備である．そこで，今回は臓器提供に対する医療機関のあり方を中心に検討する．

Key word：院内体制，臓器移植，リスクマネジメント

わが国では1997年に臓器移植法が制定[1]され，脳死下臓器提供が行われてきた．しかし年間件数で10件前後の提供数ではあまりにも少なく，現在でも臓器不全に苦しみ移植を待ち続ける命にとってはたいへん厳しい状況にある．このような現状では，国内での移植を待ち切れず海外渡航移植に踏み切らなければならない患者も存在することは十分理解できる．2009年7月，わが国では「臓器の移植に関する法律の一部を改正する法律(いわゆる改正臓器移植法)」[2]が成立し，国民の臓器移植に対する関心やマスコミ報道も大きくなった．

それでは，医療現場のどこで脳死下(あるいは心停止下)臓器提供が発生するのかといえば，それは医療機関のなかでも多忙を極める救急医療の現場が中心となる．救急医療現場では看護師をはじめ，救急医，集中治療医，脳神経外科医，麻酔科医，外科医，小児科医など多くの専門スタッフがかかわるが，通常業務でさえ多いなか，発生時にはまわりに影響する負担やリスクはさらに大きくなる．また，法改正施行により小児臓器提供や虐待，親族優先など，対応すべき問題が多く，現場での

サイドメモ　オプション提示（家族への選択肢提示）

臓器提供業務は多岐にわたり，通常診療時よりも多部署の協力が必要となる．場合によっては斡旋機関や警察との連絡など院外への協力も不可欠となる．実際に臓器提供の現場となりやすいのは，多忙を極める救命医療の現場である．臓器提供発生時には多くの人員や時間を費やすにもかかわらず，通常業務でさえ負担やリスクが大きい救急医療現場において臓器提供に対するオプション提示を要求されることも多い．オプション提示とは，医療スタッフから患者家族に対し臓器提供の意思について説明し確認することである．しかし，本来救急医療とは高度医療を施し患者の命を救命することであり，医療スタッフとしてはオプション提示に対してジレンマやその必要性について疑問を感じることも多い．法改正後，脳死下臓器提供については家族の希望だけでも可能となっており，臓器提供に対して公正・公平に関与するためには，家族との協働において脳死を含めた終末期医療に対する理解の向上が前提となる．

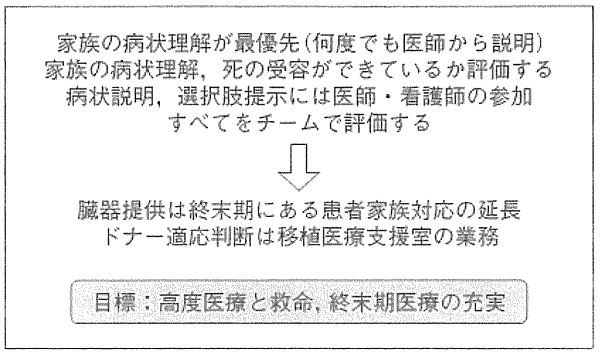

図 1　医療現場における医師と看護師の役割

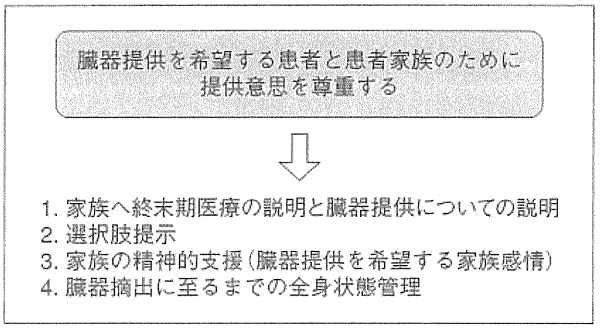

図 2　臓器提供におけるわれわれの役割

負担が増えている[3]．

また，いつ臓器提供に対する業務がはじまるのかというと，臓器提供自体を死後のことと認識している方々もいるが，実はそうではない．どのような経過であれ，入院され医療を開始した際には意思表示カード所持の確認，家族からの希望，そして医療スタッフからの選択肢提示により臓器提供への対応がはじまる．つまり臓器提供とは死亡からはじまるのではなく，あくまでも本人の意思や家族の希望によって対応がはじまるため，入院後の治療経過中においても医療スタッフの対応が必要となる．

各医療機関の立場と責任

1. 負担・リスク軽減のために臓器提供をどのようにとらえるか

脳死下，あるいは心停止下臓器提供のいずれでも“人の死”を前提とし，“死に行く者の臓器提供”があり，はじめて臓器移植が行われる．これまでの臓器提供症例においては，一部の医師あるいは医療機関により支えられてきたことは事実である．実際，すべての脳神経外科医や救急医が医療現場で積極的に臓器提供にかかわってきたわけではない．脳死診断や選択肢提示を行うことについても，日常業務の忙しさから非常に困難な状況にあるという一面があるからである．

多くの場合，医療現場で脳死あるいは全脳の機能不全を避けられない状況では，脳神経外科医や救急医は家族へ十分な病状説明を行ってから“脳死の診断”を行い，その後の治療方針を家族に問うこととなる．そのような状況では医師を含めた医療スタッフたちは病気に対する敗北や無念さを感じている[4]．もちろん，医師個人の苦悩は理解される一方で，看護師を含め医療スタッフは危機にある命のために最後まで治療を施す義務があり，死亡宣告時はもちろん出棺までは家族のためにもあらゆる努力を忘れてはならない．つまり脳死状態だからといって医療機関の努力を一方的に終わりにすべきではない．法改正を踏まえ，だれであれ医療機関に属するスタッフは本人や家族の“終末期における意思のベクトル”を能動的に問い，そのなかで臓器提供の可能性を見出していく努力が必要になる[3](図 1)．

そのなかでみえてくる課題としては，本人や家族の臓器提供意思を確認するためには情報を抽出するための選択肢提示を，だれがいつ，どのタイミングで行うのかということがあげられる．多くの施設では選択肢提示を行うべきは主治医であるとしているが，多くの医師の苦悩はここを起点としている．前述のように，とくに救急現場で医師は患者を救うために医療を施しているのである．選択肢提示は死を意味することでもあり，“手のひらを返すよう”に簡単に提示できるものではない．そこで著者らの施設では移植医療支援室を院内に設置し，臓器提供に対する終末期医療とコミュニケーションスキルを中心に救急現場の支援を行っている(図 2)．

2. 終末期医療をどのようにとらえるか

いまだに終末期医療の認識やグリーフケアは緩和ケア病棟の特別なケアとして認識されているのみであり，系統だった医学教育は少ない[5]．臓器提供症例ばかりではなく医療に取り組むには医療スタッフの終末期医療への意識，いわゆるグリーフケアの理解が重要である．