Preconditioning Donor Livers With Cromolyn or Compound 48/80 Prolongs Recipient Survival in a Rat Orthotopic Liver Transplantation Model

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ABSTRACT

Background. Acute rejection (AR) remains a challenge in organ transplantation. Preconditioning donor organs can reduce AR and prolong survival. Whether preconditioning with cromolyn (CRM), a mast cell (MC) stabilizer, or compound 48/80 (CMP 48/80), a MC degranulator, can alleviate AR and prolong survival has not been studied.

Methods. We used the male-DA-to-female-Lewis-rat orthotopic liver transplantation (OLT) model. Donors were preconditioned with CRM in a MC stabilizing way (CRM group) or CMP 48/80 in a MC depleting way (CMP 48/80 group). Rats preconditioned with phosphate-buffered saline were used as controls (PBS group). After preconditioning, OLT surgeries were carried out. OLT male-Lewis-to-female-Lewis-rats were used as the syngeneic group (syngeneic group).

Results. Rats in the PBS group developed AR rapidly and died at 7.40 ± 1.14 days. Rats in the CRM and CMP 48/80 groups had significantly slower rejections and died at day 17.40 ± 1.67 or 14.20 ± 2.28, respectively (P < .05). Rats in the syngeneic group survived more than 60 days. Rejection activity indexes (RAIs) and liver functions were all alleviated through CRM or CMP 48/80 preconditioning. Interferon-\(\gamma\) messenger RNA (mRNA) expressions were reduced and interleukin-10 mRNA levels were higher in allografts in the CRM and CMP 48/80 groups, compared with the PBS group. These were confirmed by testing serum interferon-\(\gamma\) and interlerkin-10.

Conclusion. Preconditioning donor livers with CRM or CMP 48/80 can reduce AR and prolong survival of recipients after OLT.

Liver transplantation is the most effective treatment for end-stage liver disease. Important concerns in liver transplantation include preserving organ function and prolonging recipient survival. Research has been directed at achieving such goals through donor preconditioning, preservation solution, and recipient optimization. Preconditioning donors with hypoxia [1], up-regulating heme oxygenase 1 (HO-1) [2], among others, can alleviate ischemia/reperfusion (IR) injury. IR injury affects both early- and long-term allograft function. Accumulating evidence suggests that the severity of IR injury to the allograft determines its immunogenicity. The mechanisms of promoting allograft immunogenicity are multifactorial and complex. The innate immune response, such as complement activation and up-regulation of multiple proinflammatory genes, is stimulated by IR injury. This inflammatory response, in the early stages after transplantation, is amplified by a subsequent adaptive response [3,4]. Therefore, IR injury promotes acute rejection (AR) [4,5].

M.Y. and Y.M. contributed equally to this work.

Supported by the National Natural Science Funds of China, No. 81270555; “Shu Guang Scholar” Project, Shanghai Municipal Educational Commission, No. 10SG20; and the Program for New Century Excellent Talents in University, No. NECT-13-0422.

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0041-1345/14/$ – see front matter
http://dx.doi.org/10.1016/j.transproceed.2014.01.017

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Transplantation Proceedings, 46, 1554–1559 (2014)
Mast cells (MCs) participate in IR injury in many organs, such as the heart [6] and intestine. The role of MCs in liver IR injury and the effect of preconditioning by modulating MC in liver transplantation have not been well characterized. MCs reside in the sinusoids of the liver [8] in many species (humans [9], rats [10], and canines [11]). MCs take effect mainly through releasing chemicals stored within the MC granules, which include histamine [12], interleukin [6,13], and MC tryptase [14]. There are some methods to modulate MCs. A MC stabilizer, cromolyn (CRM), and a MC degranulator, compound 48/80 (CMP 48/80; chemical formula of the monomer: C11H15NO), have previously been used to study MC function in vivo [7,15]. We demonstrated that CRM can stabilize hepatic MCs to prevent degranulation and that CMP 48/80 can deplete hepatic MCs through degranulating most of the stored granules by repeated injections [16] (data not shown).

Therefore, we aimed to investigate whether donor liver preconditioning with CRM in an MC stabilizing way or CMP 48/80 in MC depleting way would reduce AR and prolong recipient survival in a rat orthotopic liver transplantation (OLT) model.

METHODS

Ethics Statement

All animal handling and surgical procedures were approved by the Animal Care and Use Committee of the Shanghai Jiao Tong University School of Medicine.

Animals and Reagents

All animals were purchased from Sino-British Sippr/Bk Laboratory Animal Ltd. (Shanghai, China) and maintained in standard conditions with access to food and water ad libitum. Animals were fasted for the 12 hours prior to surgery. CRM and CMP 48/80 were purchased from Sigma (St. Louis, Mo, United States).

Rat OLT Surgery

Inbred male Dark Agouti (DA, RT1⁺) and female Lewis (LEW, RT1⁻) rats, aged 12–14 weeks, served as donors and recipients, respectively. Donors weighed 210–240 g, and recipients 180–210 g. Syngeneic transplantation was performed in 210–240 g male Lewis (6–8 weeks) to 180–210 g female Lewis (12–14 weeks) rats as controls. All surgical procedures were performed with clean but nonsterile instruments. Donors were anesthetized with pentobarbital sodium and recipients with ether. A nonarterIALIZED OLT was performed according to Kamada bital sodium and recipients with ether. A nonarterIALIZED OLT was performed with clean but nonsterile instruments. Donors were anesthetized with pentobarbital sodium and recipients with ether. A nonarterIALIZED OLT was performed according to Kamada

Recipient procedure, an abdominal midline incision was made. The hepatic artery was doubly ligated and divided. The bile duct was divided at the hepatic duct bifurcation. The PV and IHVC were cross-clamped. The suprahepatic vena cava (SHVC), including part of the diaphragm, was clamped with a pediatric Satinsky’s clamp. The recipient liver was removed, and the donor liver placed orthotopically. The SHVC anastomosis was performed in an end-to-end fashion with continuous 7-0 nylon suture (Johnson & Johnson Medical Shanghai Ltd., Shanghai, China). PV and IHVC anastomoses were performed using the cuff technique. The bile duct was reconstructed with an indwelling Teflon stent (inner diameter 0.6 mm; outer diameter 0.8 mm; length 8 mm).

Recipients were administered 2 mL saline via the femoral vein and 2 mL into the peritoneum intraoperatively to compensate for fluid loss, and 100,000 U penicillin intramuscularly into the hind leg. Post-transplantation, animals were placed under a heating lamp for 2 hours with free access to food and water. There were no significant differences in duration of cold preservation (<40 minutes) and portal venous clamping time (16–18 minutes) between groups.

Immunosuppressive therapy was not used. Rats that died within 3 days of transplantation were excluded from the study. Five rats in each group were kept for survival assessment.

Donor Liver Preconditioning With CRM and CMP 48/80

CRM group. CRM (100 mg/kg, intraperitoneally) was administered to DA rats 16 hours and 40 minutes prior to donor surgery as a MC stabilizing way before [18]. And it can stabilize hepatic MCs in rat liver.

CMP 48/80 group. CMP 48/80 was used to precondition DA rats, by an MC depletion method described by Wei et al. [16] We have verified that this method can deplete MCs in the rat liver as well. Briefly, a 0.1% (weight/volume) solution of CMP 48/80 in phosphate buffered saline (PBS) was administered intraperitoneally twice a day for 8 doses (0.6 mg/kg for the first 6 doses, 1.2 mg/kg for the last 2 doses) beginning with the evening dose. Donor surgery was performed 5–6 hours after the last dose.

PBS group. PBS in the same volumes as the CMP 48/80 group was given intraperitoneally as the control.

Syngeneic group. OLT male-Lewis-to-female-Lewis rats without any treatment were used as the negative control.

Sample Collection

Five transplanted rats in each group were used for sample collection. Three days after transplantation, blood samples were obtained from the vena cava after euthanasia. After coagulation, samples were centrifuged to isolate the sera, which were stored at −80°C until use. The median liver lobes were harvested and fixed in 4% paraformaldehyde in PBS for histological analysis. The left lateral segments were harvested, snap-frozen in liquid nitrogen, and stored at −80°C for RNA extraction.

Liver Enzyme Measurement

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using a Hitachi 7600-120 automatic biochemical analyzer (Tokyo, Japan). Results were expressed in international units per liter.
Pathological Examination
Livers were fixed with 4% paraformaldehyde in PBS for 24 hours and paraffin-embedded. Specimens were sectioned (4 μm thickness) and stained with hematoxylin and eosin (HE). Rejection was graded using the rejection activity index (RAI) of the Banff schema [19].

Real-Time Polymerase Chain Reaction Quantification of Gene Expressions of Cytokines in Liver Allografts
We studied the expression of interferon-γ (IFN-γ) and interleukin-10 (IL-10) in the transplanted livers. Total RNA was extracted using TRIzol reagent (Invitrogen) and quantified with absorption values of RNA samples at 260 and 280 nm. Reverse transcription was taken out with a PrimeScript RT Master Mix Perfect Real Time kit (Takara Biotechnology, Dalian, China). Complementary DNA (cDNA) was stored at −80°C until use. All samples were measured with a SYBR Premix Ex Taq (Takara Biotechnology, Dalian, China) real-time polymerase chain reaction (PCR) kit. The primers of cytokines were as follows: IFN-γ, 5'-CCC TCT GGC TGT TAC TGC-3' (forward) and 5'-CTT CT-3' (reverse); IL-10, 5'-TGCTT TTC CTT CGC TTC CTT AGG-3' (reverse); IL-10, 5'-AACCT ATT CAT GGC TCC AGT-3' (reverse); β-actin, 5'-CCC GCG GAG TAC AAC CCC GAG TAC AAC CTT CT-3' (forward) and 5'-CGT CAT CCA TGG CGA ACT-3' (reverse). The assays were done according to the manufacturer’s standard procedure with an ABI 7500 Fast Real-Time PCR system (Applied Biosystems Inc., Foster City, United States). The standard procedure was incubation (95°C for 30 seconds), 40 cycles of denaturation (95°C for 5 seconds), annealing, and extension (60°C for 1 minute). IFN-γ and IL-10 gene expression was normalized to β-actin. Each experiment was performed in triplicate. The copies of relative messenger RNA (mRNA) were calculated as described previously [20].

Serum Cytokine Measurements
Serum levels of IFN-γ and IL-10 were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Senxiang Science and Technology Co., Shanghai, China). The assay was performed according to the manufacturer’s instructions.

Statistical Analysis
Values are expressed as the mean ± SD and analyses were performed with the SPSS statistical package (SPSS 19.0 for Windows; SPSS, Inc., Chicago, Ill, United States). Kaplan-Meier analysis was used to evaluate graft survival; the Mann-Whitney U test was used for estimation of survival difference among groups. Analyses of RAIs, liver function assays, real-time PCR, and ELISA were performed with analysis of variance (ANOVA) along with Dunnett post-test. A P value of < .05 was considered to be statistically significant.

RESULTS
CRM or CMP 48/80 Alleviated AR as Measured by Survivals and RAI
Female Lewis rats transplanted with hepatic allografts from DA rats survived a mean of 7.40 ± 1.14 days. Pretreatment with CRM or CMP 48/80 significantly prolonged survivals (17.40 ± 1.67 and 14.20 ± 2.28 days, respectively; P < .05). Control male Lewis-to-female Lewis isograft rats survived longer than 60 days (Fig 1).

DISCUSSION
In organ transplantation, attenuating transplanted organ injury and prolonging recipient survival is critical. University of Wisconsin (UW) solution and tacrolimus-based immunosuppression were two important milestones in the history of liver transplantation [21]. Recipient-based immunosuppression therapy has many complications, such as infection and virus-related malignancies [22]. Although UW solution has improved liver preservation, IR injury remains a
significant challenge. Reducing IR injury and inhibiting donor-specific immunologic response are the focuses of research for improving survival in liver transplantation.

Experimental animal models of OLT play a crucial role in liver transplantation research. Rat or mouse OLT models are most frequently used, especially the DA-to-Lewis rat OLT model in AR research [23,24]. The male-DA-to-female-Lewis rat OLT model exhibited AR and mean survival days in the PBS-treated group were 7.40 ± 1.14 days, shorter than in the male-DA-to-male-Lewis rats in previous publications [23,25]. This finding may be due to a minor histocompatibility antigen (H-Y antigen), verified to promote rejection in male-to-female gender-mismatched transplantation [26–29].

Th1/Th2 cytokine expression is closely connected to transplantation rejection and tolerance. A representative Th-1 cytokine (IFN-γ) was found to increase transplantation rejection, whereas a representative Th-2 cytokine (IL-10) is important for allograft acceptance [30]. In our study, real-time PCR demonstrated IFN-γ mRNA levels to be lower in rats treated with CRM and CMP 48/80, whereas IL-10 mRNA levels were higher. Cytokines expression was confirmed based on serum ELISA results. These results
indicated that the AR reduction and prolonging of survival correlate with Th1/Th2 balance shifting.

How can CRM and CMP 48/80 preconditioning shift the Th1/Th2 balance, reduce the risk of AR, and prolong survival? CRM is a well-known MC stabilizer, which has been studied both in vivo and in vitro to understand the function of MCs [7,31,32]. Preconditioning with CRM can alleviate organ IR injury [7,33,34]; CMP 48/80 is largely used as a MC degranulator [35], although it has other effects as well [36]. Repeated injection of CMP 48/80 can deplete MCs in the skin [16], as well as the liver (data not show). In our study, donor liver preconditioning with CRM in a MC stabilizing way or CMP 48/80 in a MC depleting way can reduce rejection and prolong survival. In fact, the effects of stabilizing MCs with CRM or depleting MCs with CMP 48/80 may be almost the same in this study, namely, a lesser amount of chemicals was released after transplantation by MCs. The two methods arrived at such a goal in different ways: one prevents MC releasing chemicals after transplantation by stabilizing them with CRM; the other is exhausting most chemicals before transplantation by depleting MCs with CMP 48/80. As a result, a lesser amount of chemicals was left and could be released during transplantation. Because the chemicals releasing from MC degranulation promote IR injury in many organs [7,33,34], and IR injury promotes AR, it may indicate that MCs may play a role in hepatic IR injury. This requires further research.

While our findings may result in the use of these drugs in the clinical settings, CRM and CMP 48/80 cannot be used directly. It has been previously shown that modifying UW solution by adding chemicals promotes organ preservation [37]. Whether adding CRM into UW solution to modulate MCs in the liver may exert the same effect requires further study.

In conclusion, preconditioning with CRM or CMP 48/80 can attenuate acute rejection and prolong recipient survival. These results may be helpful in their development as new drugs in liver transplantation.

ACKNOWLEDGMENTS
We acknowledge Jian Zhang and Li-song Shen for the liver function analysis, Mei-ping Shen for rat housing, and Yi Liu for assistance with pathology.

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