Experimental and Clinical Significance of Antinuclear Antibodies in Liver Transplantation

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Histone H1 and high-mobility group box 1 (HMGB1) proteins are known to initiate an immune reaction, and the corresponding antibodies (Abs) possess immunosuppressive activity. In the present study, we aimed to evaluate the immunological role of antinuclear Abs in experimental and clinical liver transplantation. In a rat tolerogenic orthotopic liver transplantation (OLT) model, antihistone H1 and HMGB1 titers were induced during the rejection and tolerance induction phases, respectively. Those Ab responses also were confirmed in a drug-induced tolerance model (acute rejection model + cyclosporin A [0 to 14 days after OLT]). We also found a similar tendency in our clinical drug-free patient (who experienced complete cessation of any immunosuppressive treatments) and that antinuclear Abs induced in the serum after cessation of immunosuppressants play a part of immune privilege in this patient. These results suggest that antinuclear Abs are important factors for overcoming rejection and the subsequent tolerance induction in liver transplantation.

Keywords: Histone H1, HMGB1, Antinuclear antibody, Liver transplantation, Tolerance.

In a rat tolerogenic orthotopic liver transplantation (OLT) model (donor DA to recipient PVG [DA-PVG] combinations), allograft rejection is naturally overcome without the use of immunosuppressant, and spontaneous tolerance is induced. It is known that the serum of PVG-recipient rats bearing DA livers (postOLT serum) has strong immunosuppressive activity after OLT (1, 2), suggesting that the identification of natural humoral factors from the postOLT serum could aid in the development of novel immunosuppressants and/or diagnostic markers. Recently, we provided evidence that autoreactive antibodies (Abs) against nuclear histone H1 are a major immunosuppressive factor induced in early postOLT serum (3, 4). Histone H1 normally binds to the linker DNA between nucleosomes and is involved in the formation of higher-order chromatin structures (5). Histone H1 possesses various important functions, including a role in transmitting apoptotic signals from the nucleus to the mitochondria, which release apoptogenic factors into the cytoplasm, after DNA double-strand breaks (6) and in normal dendritic cell (DC) differentiation, on the basis of the evidence that the production and differentiation of DCs in histone H1-deficient mice were reduced significantly (7).

However, nuclear-binding protein high-mobility group box 1 protein (HMGB1), which previously was thought to function only as a nuclear factor that enhances transcription, recently was discovered to be a crucial cytokine that mediates the response to infection, injury, and inflammation (8). HMGB1 is passively released from necrotic cells; however, some cell types, including monocytes and macrophages, can secrete HMGB1 actively without dying (9–11). Exogenous HMGB1 effectively controls DC maturation and function. Also, interestingly, anti-HMGB1 Ab and Ab against the HMGB1 receptor, which is known as the receptor for advanced glycation end products (RAGE), possess significant immunosuppressive activity in vitro (9). However, the essential roles of nuclear antigens such as histone H1 and HMGB1 and the corresponding autoreactive Ab in

transplant immunology have not yet been clarified. In this study, we investigated the significance of antinuclear Abs for overcoming rejection and the subsequent tolerance induction in experimental and clinical liver transplantation.

To establish the experimental liver transplantation models in rats, OLT was conducted using a previously described technique (*12*) in the following combinations: DA (RT1[a]) to PVG (RT1[c]) (DA-PVG), DA to LEW (RT1[l]) (DA-LEW), and DA-LEW with a cyclosporin A (CsA, Novartis, Basel, Switzerland, 15 mg/kg/day, 0–14 days after OLT). OLT (DA-PVG) rats naturally overcome the rejection reaction without any immunosuppressant and naturally induce tolerance (tolerogenic OLT model). OLT (DA-LEW) rats die from acute rejection within 14 days and were designated as an acute rejection model. OLT (DA-LEW) rats treated with CsA can survive for a long time and were designated as a drug-induced tolerance model.

In our clinical cases of liver transplantation, one patient was completely weaned off of the immunosuppressant. In brief, a biliary atresia patient underwent the Kasai operation at 2 months of age (1990). In 1994, the patient underwent living related liver transplantation (LRLT) and had been treated with an immunosuppressive regimen consisting of CsA and steroid for 5 years up to September 1999, when the patient suffered from post-transplant lymphoproliferative disease (PTLD). As the treatment for PTLD, this patient's immunosuppressive regimen was abandoned, and the patient was treated with chemotherapy, which was successful. Since this patient overcame PTLD in 1999, the patient has not required immunosuppressants and has not had a rejection reaction for liver allografts until now. Sera were collected from 1994 to 2004. As controls, healthy volunteers were used, all of whom were liver transplantation donors. Informed consent was obtained from all participants either directly from the patients and/or from their parents, and ethics approval was obtained from the Chang Gung Memorial Hospital.

To evaluate the histone H1/HMGB1-specific Ab titer, we used enzyme-linked immunosorbent assay (ELISA) (*3*). In brief, calf thymus histone H1 (Upstate, Charlottesville, VA) or recombinant HMGB1 (Sigma, St. Louis, MO) was coated onto a 96-well microtiter plate (Nalge Nunc International, Roskilde, Denmark) by incubation at room temperature for 1 hr. After blocking the plate, serum samples (50 μL, ×50 dilution) were added to the wells and incubated at room temperature for 1 hr. Then, secondary peroxidase-conjugated anti-rat (Biosource International, Camarillo, CA) or human IgG (CHEMICON International, Temecula, CA) was added and incubated at room temperature for 1 hr, followed by the addition of substrate solution. The absorbance (405 nm) was then measured using an MRX Microplate Reader (Dynex Technologies, Chantilly, VA). The cut-off for seropositivity was determined as the mean+3 standard deviation (SD) of the levels of the negative control group (*P*<0.01).

To evaluate the immunological role of antinuclear Abs in the serum from a drug-free patient, neutralized antigens (histone H1 and HMGB1) and postOLT serum were simultaneously added in the culture of mixed lymphocyte reaction (MLR) as previously described (*3*). Statistical analysis was performed using Student's *t* test.

We first checked the antinuclear Ab titer in a rat tolerogenic OLT model (DA-PVG). As shown in Figure 1, the anti-
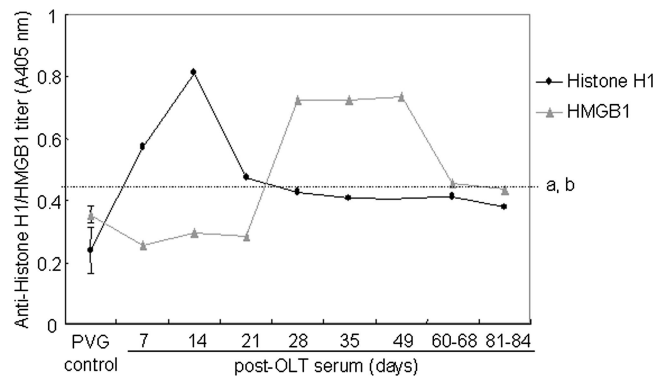
FIGURE 1. Nuclear antigen (histone H1 and HMGB1)-specific Ab response after OLT in a DA-PVG natural tolerance model. Anti-histone H1/HMGB1 titer (A405 nm) was measured by ELISA. PostOLT serum at 7 (n=8), 14 (n=8), 21 (n=3), 28 (n=3), 35 (n=4), 49 (n=3), 60 to 68 (n=5), and 81 to 84 (n=3) days in a natural tolerance model. Dotted line shows the cut-off line (mean+3SD of the levels of the naïve PVG serum, n = 5; [a: histone H1; b: HMGB1]).

histone H1 titer was transiently increased at the rejection phase after OLT (days 7–21), a finding that is similar to the results of a previous report (*3*, *4*). Additionally, the anti-HMGB1 titer was significantly up-regulated at the tolerance induction phase (days 28–49) in place of anti-histone H1 Ab. These results suggest that the appearance of antinuclear Abs may be an important phenomenon and may be indispensable for natural tolerance induction to occur in a rat tolerogenic OLT model.

To further explore the significance of the Ab response against nuclear proteins exposed in necrotic cells triggered *via* rejection, we next measured the antinuclear Ab titer both in a DA-LEW acute rejection model and in a drug-induced tolerance model (DA-LEW+CsA). As shown in Figure 2, the antihistone H1 titer of a drug-induced tolerance model under CsA treatment was significantly greater than that of a DA-
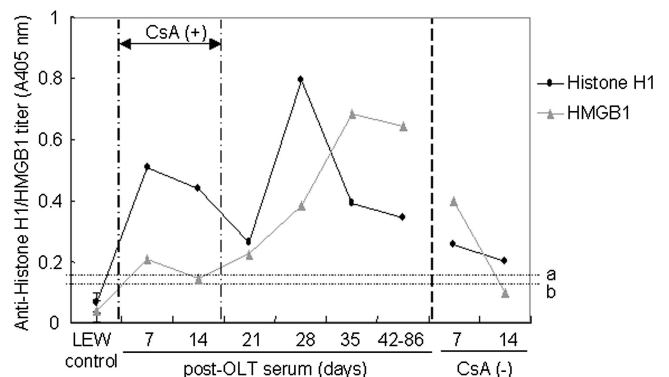
FIGURE 2. Nuclear antigen (histone H1 and HMGB1)-specific Ab response after OLT in a DA-LEW+CsA drug-induced tolerance model and a DA-LEW acute rejection model. PostOLT serum at 7 (n=5), 14 (n=4), 21 (n=3), 28 (n=4), 35 (n=3), and 42 to 86 (n=4) days in a drug-induced tolerance model and 7 (n=4) and 14 (n=3) days in an acute rejection model. Dotted line shows the cut-off line (mean+3SD of the levels of the naïve LEW serum, n=5 [a: histone H1; b: HMGB1]).

LEW rejection model (days 7 and 14) and that of naïve LEW rats, and it was further up-regulated after the cessation of CsA (day 28). Similarly, in a DA-PVG natural tolerance model, anti-histone H1 titer was down-regulated at the latter phase after OLT, whereas the titer was still greater than that of naïve LEW rats. However, anti-HMGB1 titer was gradually and significantly up-regulated after the cessation of CsA in a drug-induced tolerance model. These results suggest that antinuclear Abs are actively expressed after tolerance induction and to overcome the rejection episodes both in a natural tolerance model and in a drug-induced tolerance model.

To evaluate the significance of antinuclear Abs in clinical liver transplantation, we next picked up serum samples from the drug-free patient (who experienced complete cessation of immunosuppressants) and other LRLT patients (who were under immunosuppressants). As shown in Figure 3A, the antihistone H1/HMGB1 titer of this drug-free patient was dramatically greater than those of healthy volunteers before

LRLT. Even after LRLT, the antihistone H1 titer remained at a high level (at least for 4 years), whereas those of other transplant patients, including patients who suffered from liver cirrhosis, biliary atresia, and neonatal hepatitis, as well as the levels observed after LRLT, were immediately down-regulated to the control level or less (Fig. 3B). However, anti-HMGB1 titer was gradually down-regulated to the control level within 4 years in this drug-free patient. Interestingly, antihistone H1 titer was dramatically decreased before this drug-free patient suffered from PTLD. After chemotherapy and the cessation of immunosuppressants (CsA and steroid) successfully treated the PTLD, the liver enzymes (AST and ALT) were transiently increased (*13*) and, subsequently, the antihistone H1/HMGB1 titer was up-regulated. We speculate that liver damage after cessation of CsA may induce the exposition of nuclear antigens and that the subsequent production of antinuclear Abs in this drug-free patient. Intriguingly, antinuclear Abs were maintained at a high level continuously
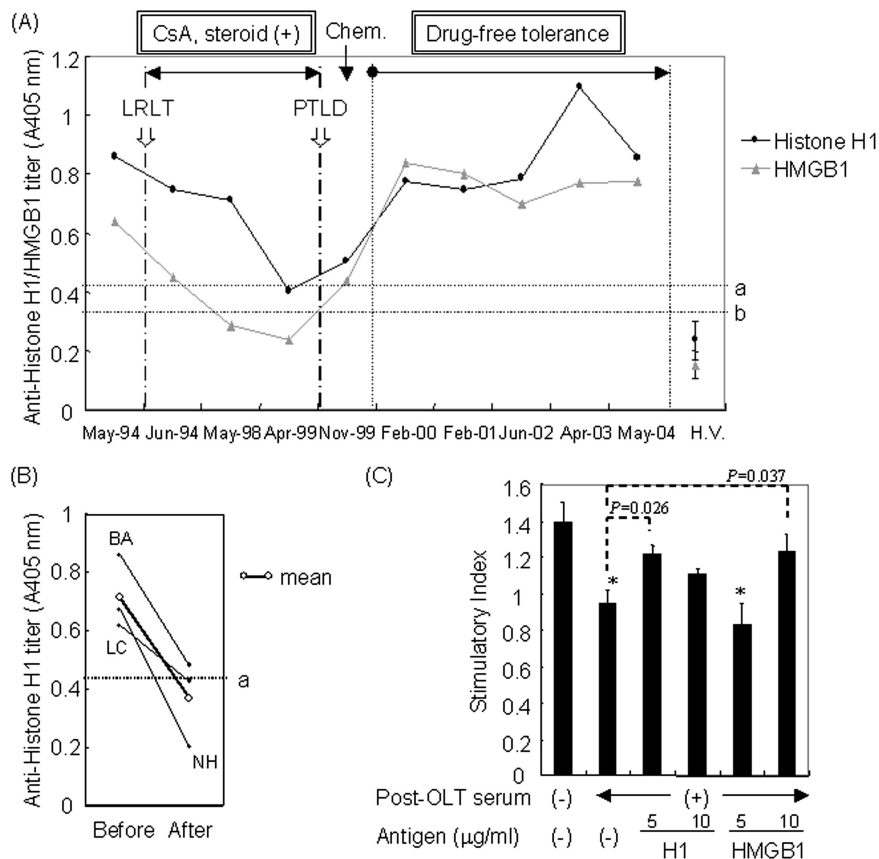
FIGURE 3. (A) Nuclear antigen (histone H1 and HMGB1)-specific Ab response after LRLT in a drug-free patient. Dotted line shows the cut-off line (mean+3SD of the levels of the control serum (H.V.: healthy volunteer, n=5 [a: histone H1; b: HMGB1]). LRLT, living related liver transplantation; PTLD, posttransplant lymphoproliferative disease; Chem., chemotherapy (6 courses: methotraxate, Ara-C, cyclophosphamide, vincristine, adriamycin, and acyclorir). (B) Histone H1-specific Ab response before/after LRLT (day 7) in LRLT patients suffering from liver cirrhosis (LC, n=18), biliary atresia (BA, n=17) and neonatal hepatitis (NH, n=4). (C) Neutralization of antinuclear Abs reduced the immunosuppressive activity of postOLT serum from a drug-free patient after cessation of immunosuppressants. Mitomycin C-treated stimulator (DA) and responder (LEW) cells (each 2×10^5 cells in 100 μl) were mixed and cultured for 84 hr. PostOLT serum was added at a final concentration of 1% to evaluate the MLR-inhibitory activity. Simultaneously, exogenous histone H1 and HMGB1 were added at up to 10 μg/ml to neutralize the antihistone H1 or HMGB1 Abs in the postOLT serum. The allogeneic T-cell response was determined using a Cell Proliferation ELISA, BrdU (Roche Diagnostics, Mannheim, Germany) with an MRX Microplate Reader (Dynex Technologies). Stimulatory Index=(BrdU incorporation of allogeneic combination [DA/LEW])/(BrdU incorporation of syngeneic combination [LEW/LEW]). *Significantly inhibited as compared with postOLT serum (−) (*P*<0.05).

during "drug-free tolerance" (from 1999 to the present), similar to a drug-induced tolerance model. We also demonstrated that postOLT serum after cessation of CsA possessed immunosuppressive activity in vitro in this drug-free patient (*13*, Figure 3C), whereas its MLR-inhibitory activity was partly reduced by the effect of neutralized antigens. These results suggest that the existence of antinuclear Abs in the systemic circulation may regulate uncontrollable immune responses such as acute/chronic rejection after LRLT or PTLD caused by excessive immunosuppressive treatments.

Clinical evidence suggests that antinuclear autoreactive Abs are associated with the pathogenesis of autoimmune disorders (*14*, *15*). However, anti-histone H1/HMGB1 Abs possess strong immunosuppressive activity (*3*, *4*, *9*). Recently, we demonstrated that overcoming rejection is related to the induction of immature DCs and that the inhibition of the activity of natural killer cells through autoimmune regulation by the blockage of histone H1 (*16*). Similarly, anti-HMGB1 Ab regulates the expression of CD80, 83, and 86 on DCs and possesses immunosuppressive activity in vitro (*9*). These results suggest that antinuclear Abs may suppress cell-to-cell interaction between antigen-presenting cells and T cells and that a spontaneous or active autoimmune response against nuclear antigens such as histone H1 and HMGB1 may be indispensable for overcoming acute rejection and for promoting the subsequent tolerance induction. Further investigations are currently underway to elucidate the functional role of antinuclear Abs in both cellular and humoral immunity.

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