Impact of vaccine therapy using nuclear histone H1 on allograft survival in experimental organ transplantation

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Abstract

Background: We recently reported that autoreactive antibody (Ab) against nuclear histone H1 had been identified as an immunosuppressive factor in a rat tolerogenic orthotopic liver transplantation (OLT) model. The present study aimed to determine whether the up-regulation of antihistone H1 Ab by histone H1 vaccination leads to tolerance.

Methods: Histone H1-immunized rats were established by intraperitoneal vaccination with histone H1 at every two-weekly interval. By using mixed lymphocyte reaction (MLR) and heterotopic heart transplantation (HHT), the alloreactive T cell response and allograft survival of histone H1-immunized rats were compared with those of control rats. Cytokine and cellular profiles in histone H1-immunized rats were determined by reverse transcriptase polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA) and flow cytometry.

Results: Immunization with histone H1 in Freund’s adjuvant induced alloreactive T cell unresponsiveness and prolonged heterotopic heart allograft survival. It also down-regulated the expression of major histocompatibility complex (MHC) class II and CD25 on splenic cells, elevated the T helper cell type 2 (Th2) skewing index (Interleukin (IL)-4/interferon (IFN)-γ ratio or IL-4/IL-2 ratio) and modified the serum cytokine profiles.

Conclusions: The present results suggest that histone H1 vaccination of transplant recipients, which leads to the production of immunosuppressive factor and the modification of the cytokine/cellular profiles, has great potential as a tolerance therapy for prospective transplantation.

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1. Introduction

In a rat tolerogenic orthotopic liver transplantation (OLT) model (DA-PVG combinations), it is known that the recipient serum after OLT (post-OLT serum) shows immunosuppressive activity [1,2], suggesting that the humoral factors from the post-
OLT sera would be useful in the development of novel immunosuppressants. Several candidates for these immunosuppressive factors in post-OLT serum have been identified, including soluble donor-specific major histocompatibility complex (MHC) class I molecules [3], anti-donor MHC class II antibodies (Abs) [4], liver suppressor factor-1 (LSF-1) (40 kDa) [5,6], which was induced in the serum of OLT rats, and LSF-2 (87 kDa)/LSF-3 (10 kDa), which was induced in the serum of re-OLT rats [7].

In our previous reports, we have provided evidence that autoreactive Abs against histone H1 are a major immunosuppressive factor induced in early post-OLT serum [8,9]. Histone H1 normally binds to the linker DNA between nucleosomes, and is involved in the formation of higher-order chromatin structures [10]. It is also known that histone H1 carries out various important functions. Konishi et al. report that histone H1.2 plays an important role in transmitting apoptotic signals from the nucleus to the mitochondria, which release apoptogenic factors into the cytoplasm following DNA double-strand breaks [11]. Furthermore, histone H1 appears to be an important factor in the differentiation of normal dendritic cells (DCs), based on evidence that the production and differentiation of DCs in histone H1-deficient mice were significantly reduced [12]. Our previous study also suggests that the blockade of histone H1 modulates DCs toward tolerogenic status, decreases the cytotoxicity of lymphokine-activated killer (LAK) and human histone H1 modulates DCs toward tolerogenic status, decreases the cytotoxicity of lymphokine-activated killer (LAK) and human histone H1-autoantibodies do in fact play a role in tolerance induction.

2. Objective

The present study aimed to determine whether the up-regulation of antihistone H1 Ab by histone H1 vaccination leads to tolerance. In the present study, we established histone H1-immunized rats by histone H1 vaccination and demonstrated that the up-regulation of antihistone H1 Ab induces alloreactive T cell unresponsiveness both in vitro and in vivo.

3. Materials and methods

3.1. Animals and immunization

Male DA (MHC haplotype RT1*) and LEW (RT1*) rats, each 4 weeks of age, were obtained from Japan SLC (Hamamatsu, Japan) and National Animal Breeding Center (Taipei, Taiwan), respectively. All animals were maintained in specific pathogen-free animal facilities with water and commercial rat food provided ad libitum.

The LEW rats were immunized intraperitoneally every 2 weeks with calf thymus histone H1 (Upstate, Charlestown, MA; 100 μg) which had been preadsorbed with Freund’s complete adjuvant (FCA) (1st immunization; Wako, Osaka, Japan) or Freund’s incomplete adjuvant (FIA) (2nd immunization; Wako). For a final boost, LEW rats were immunized with histone H1 (100 μg) without adjuvant. Control rats were immunized with phosphate buffered saline (PBS) or bovine serum albumin (BSA) on the same schedule as histone H1-immunized rats.

3.2. Measurement of antihistone H1 titer by enzyme-linked immunosorbent assay (ELISA)

In order to analyze histone H1-specific autoantibody titer, calf thymus histone H1 (Upstate) was coated at 20 μg/ml in 100 mM NaHCO3 (pH 9.3) onto a 96-well microtiter plate (Nalge Nunc International, Roskilde, Denmark) by incubation at room temperature for 1 h. For measurement of anti-BSA titer, BSA was coated at 20 μg/ml instead of histone H1. After blocking the plate, serum samples (50 μl dilution with 10 mM Tris–HCl (pH 8.0), 0.9% (w/v) NaCl, 0.5% (w/v) Tween 20) were added to the wells and incubated at room temperature for 1 h. For serum samples, secondary peroxidase-conjugated anti-rat IgG (x2000 dilution, Biosource International, Camarillo, CA) was added and the mixture was incubated at room temperature for 1 h, followed by the addition of 2,2'-azo-bis(3-ethylbenzothiazolin-6-sulfonic acid) (ABTS) substrate solution (Sigma, St. Louis, MO) for enzyme-linked immunosorbent assay (ELISA). Absorbance at 405 nm was then measured using an MRX Microplate Reader (Dynex Technologies, Chantilly, VA).

3.3. Mixed lymphocyte reaction (MLR) and heterotopic heart transplantation (HHT)

After administering the final boost for 3 days, mixed lymphocyte reaction (MLR) and heterotopic heart transplantation (HHT) were performed as previously described [2,8]. For MLR, responder LEW cells (5x10^6 cells in 100 μl) derived from histone H1- or PBS-immunized rats were mixed with stimulator DA cells (8x10^5 cells in 100 μl) in 96-well round-bottom plates (Nalge Nunc International) and incubated at 37 °C for 84 h in a humidified atmosphere of 5% CO2/95% air. Cell proliferation was determined using a BrdU Labeling and Detection Kit III (Roche Molecular Biochemicals, Mannheim, Germany) with an MRX Microplate Reader (Dynex Technologies).

For HHT, hearts from DA rats were heterotopically transplanted into the neck of histone H1-, BSA- or PBS-immunized LEW rats. A sham control group was treated with saline.

3.4. Flow cytometry

After vaccination with histone H1 or BSA, LEW splenocytes were harvested and the cells (10^6 cells/100 μl) were incubated with mouse anti-rat CD32 (Fcγ II receptor) (BD Biosciences, San Jose, CA) in a staining buffer (1% FCS/0.1% NaN3/PBS) at 4 °C for 15 min to block non-antigen-specific binding of immunoglobulins. The cells were then incubated at 4 °C for 30 min with fluorescein isothiocyanate (FITC)-, or phycoerythrin (PE)-conjugated anti-specific Abs against CD4, CD8, CD25, CD45RA, CD62 or MHC class II (1 μg, BD Biosciences or Immunotech, Marseille, France). After being washed twice...
each, the cell samples were analyzed by an EPICS® ALTRA™ Flow Cytometer (Beckman Coulter, Miami, FL) using EXPO32 software.

3.5. mRNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was extracted from splenocytes derived from naïve, histone H1- or control (PBS or BSA)-immunized rats using TRIzol® reagent (GIBCO™ Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. cDNA was synthesized from total RNA employing ImProm-II™ Reverse Transcriptase (Promega Biosciences, Inc., San Luis Obispo, CA) following the manufacturer’s instructions. The cDNA were then amplified by polymerase chain reaction (PCR) in a total volume of 50 μl containing 3 μl of cDNA, 1 μl of the 10-μM PCR primer, 5 μl of 25-mM dNTP, 5 μl of 10-fold PCR buffer, 35.5 μl of H2O, and 0.5 U Taq enzyme (Roche Molecular Biochemicals). The rat-specific PCR primers are shown in Table 1. The PCR reaction was hot-started at 94 °C for 2 min to denature all cDNA samples. After the initial denaturation step, the cDNA samples were subjected to rounds of denaturation (94 °C for 40 s), annealing at 55 °C to 60 °C for 30 s, and extension at 72 °C for 30 s. Each reaction was completed by heating at 72 °C for the final 10 min. The samples were amplified with different cycle numbers based on optimal amplification efficiencies: 26 (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)), 22 (interferon (IFN)-γ and interleukin (IL)-2) and 30 (IL-4). The amplification products were separated by electrophoresis on 2% TBE agarose gels and stained with ethidium bromide. The gel profiles were visualized (photographed) with ultraviolet illumination gel documentation (UVisitec, Cambridge, UK) and analyzed with ultraviolet illumination photo version 99 (UViitec) and TotalLab software version 1.00 (Nonlinear USA, Inc., Durham, NC). PCR products were titrated to establish standard curves for documenting linearity and permitting semiquantitative analysis. Levels of gene expression were expressed as the ratios of densities between PCR products and GAPDH in the same sample. All experiments were repeated three times.

3.6. Measurement of serum cytokines by sandwich ELISA

Sera were collected at every 2 weeks after antigen-immunization and were assayed in duplicate for the presence of cytokines (IL-2, IFN-γ, IL-4, IL-10 and tumor necrosis factor (TNF)-α) using Endogen® Rat cytokine ELISA kits.

Fig. 1. Antihistone H1/BSA titer after histone H1/BSA vaccine therapy. (A) Antihistone H1 titer or (B) anti-BSA titer (optical density at A405 nm) was measured by ELISA as described in the Materials and methods section. Histone H1-immunized rats (antihistone H1 titer): n=20; PBS-immunized rats (antihistone H1 titer): n=6; BSA-immunized rats (anti-BSA titer): n=6.

Fig. 2. Histone H1 vaccine therapy induces allogeneic T cell unresponsiveness both in vitro (one-way MLR) and in vivo (HHT). (A) MMC-treated stimulator (DA) and responder (LEW) cells (gray bar: histone H1-immunized rats; black bar: PBS-immunized rats) were mixed (Stimulator/Responder ratio=16:1) and cultured for 84 h. The proliferation of responder cells (BrdU incorporation) was determined using a BrdU Labeling and Detection Kit III (optical density at A405 nm). The bars indicate the average absorbance values from three individual rats. *Significantly low compared with control PBS group (P<0.05). (B) The DA allograft survival of histone H1-immunized LEW rats (n=11) was significantly prolonged (P<0.005) compared with that of sham control recipients (saline treatment rats: n=10; BSA-immunized rats: n=3; PBS-immunized rats: n=3).
we continued vaccination until it reached plateau level at a total of 10 immunizations. We also established PBS- and BSA-immunized rats as a control group to rule out the effect of adjuvant and histone H1-unrelated Ab response. In control BSA-immunized rats, anti-BSA titer was dramatically increased after 2 immunizations (Fig. 1B), but we continued vaccination as in the case of histone H1-immunized rats.

4.2. The effect of histone H1 immunization on in vitro and in vivo alloreactivity

After the final boost, the splenocytes of control PBS- or histone H1-immunized LEW rats were mixed with stimulator DA rat splenocytes for in vitro testing (one-way MLR). As shown in Fig. 2A, the allogenic T cell response of histone H1-immunized LEW rats was significantly low \( (P < 0.05) \) compared with that of control PBS-immunized rats.

Subsequently, we applied the histone H1-, PBS-, and BSA-immunized LEW rats as recipients for in vivo testing (HHT). As shown in Fig. 2B, the DA heart allograft survival of histone H1-immunized LEW rats was significantly prolonged (mean±S.D.=22.2±12.0, \( n=11 \), \( P=0.005 \) as compared with the saline group), while all control counterparts rejected the heart allograft within 14 days (saline treatment: mean±S.D.=7.5±1.2, \( n=10 \); BSA+adjuvant: mean±S.D.=10.7±2.9, \( n=3 \); PBS+adjuvant: mean±S.D.=5.3±4.0, \( n=3 \)).

### 4. Results

#### 4.1. Establishment of histone H1-immunized rats

To evaluate the effect of histone H1 vaccination under our MLR conditions (stimulator DA, responder LEW splenocytes), we attempted to establish histone H1-immunized LEW rats. As shown in Fig. 1A, antihistone H1 titer reached its highest level at 7 immunizations, and
4.3. Cellular profiles after histone H1 vaccination

In order to explore the effects of histone H1 vaccination in immune cells, we next checked the cell surface markers of splenocytes derived from histone H1- or control BSA-immunized rats (n=3) after the final immunization. As shown in Fig. 3, the mean fluorescence intensity of splenocytes in histone H1-immunized rats expressed lower levels of MHC class II and CD25 as compared with control BSA-immunized rats, whereas other markers (CD4, CD8, CD45RA and OX62) on splenic cells in histone H1-immunized rats were comparable to control counterparts (data not shown).

4.4. Cytokine gene expression after histone H1 vaccination

To explore the effects of histone H1 vaccination on serum cytokine profiles, we next measured serum IL-2, IFN-γ, IL-4, IL-10 and TNF-α levels during histone H1 vaccination. Using our assay system, however, we were unable to confirm the levels of IL-2 and IL-4 in naïve LEW rats and histone H1- or BSA-immunized models during vaccination (data not shown). As shown in Table 2, the serum IFN-γ level of histone H1-immunized rats was significantly higher than that of naïve or control-immunized rats (P<0.05).

4.5. Serum cytokine profiles during histone H1 vaccination

To explore the effects of histone H1 vaccination on serum cytokine profiles, we next measured serum IL-2, IFN-γ, IL-4, IL-10 and TNF-α levels during histone H1 vaccination. Using our assay system, however, we were unable to confirm the levels of IL-2 and IL-4 in naïve LEW rats and histone H1- or BSA-immunized models during vaccination (data not shown). As shown in Table 2, the serum IFN-γ level of histone H1-immunized rats was significantly higher than that of naïve LEW rats, and interestingly, the serum IL-10 level of histone H1-immunized rats was transiently up-regulated when the titer of antihistone H1 Ab was increasing in the serum (Fig. 1A). On the other hand, the serum TNF-α level was dramatically up-regulated when antihistone H1 titer reached plateau level.

5. Discussion

In the present study, we demonstrated that up-regulation of antihistone H1 Ab by histone H1 vaccination induces alloreactive T cell unresponsiveness in MLR (Fig. 2A) and prolongs the allograft survival in HHT (Fig. 2B). In our histone H1-immunized rats, however, the survival days of the donor heart graft were limited (mean ± S.D. = 22.2 ± 12.0). The antihistone H1 titer of histone H1-immunized rats remained at a high level for at least 2–3 weeks after the final boost of histone H1 (3 days before HHT) and gradually down-regulated (data not shown). Therefore, graft survival would be further prolonged if we continued histone H1 vaccine therapy until rejection was overcome and tolerance induction was fully established.

We also provide evidence that histone H1 vaccine therapy induces the down-regulation of both MHC class II and CD25 molecules (Fig. 3). MHC class II and CD25 (IL-2 receptor) molecules are essential for allore cognition and the subsequent immune responses [14–16], and the blockade of these molecules by anti-MHC class II monoclonal Ab and anti-CD25 monoclonal Ab (basiliximab) is known to prevent allograft or xenograft rejection not only in experimental organ transplantation but also in clinical settings [17–24]. Furthermore, we showed the elevation of IL-4/IFN-γ ratio and IL-4/IL-2 ratio in splenocytes after histone H1 vaccination (Fig. 4), suggesting that the up-regulation of antihistone H1 Abs induced Th2-type responses. In experimental and clinical transplantation, intriguingly, the physiological balance toward a Th2 cytokine profile predisposes to improved graft acceptance [25–28]. In the cytokine paradigm of histone H1-immunized rats, we showed the up-regulation of serum IFN-γ and IL-10 levels during histone H1 vaccination (Table 2). It is well known that IL-10 has strong anti-inflammatory activities and acts as a general suppressive factor of immune reactions, as well as strongly down-regulating MHC class II on antigen-presenting cells [29,30]. Klyushnenkova et al. report that the suppression of MLR by human mesenchymal stem cells is mediated by the secretion of IFN-γ and IL-10 [31]. Additionally, our previous study suggests that antihistone H1 Abs lead to the induction of regulatory T cells and immature DCs and the inhibition of the activity of NK cells during the rejection phase in a rat tolerogenic OLT model [13]. Further investigation is currently underway to elucidate the immunosuppressive mechanisms of antihistone H1 Abs both in cellular and humoral immunity.

In addition to the present examination, it is important to substantially analyze the possible side effects of histone H1 vaccine therapy prior to its clinical application because clinical evidence suggests that antinuclear autoreactive Abs are associated with the pathogenesis of autoimmune disorders [32,33]. In our animal models of histone H1 vaccine therapy, no adverse effects of histone H1 immunization were observed in weight or appearance or in biochemical examinations in comparison with those of control counterparts (data not shown). However, we confirmed in the present study that the serum TNF-α level was dramatically up-regulated when antihistone H1 titer reached plateau level (Fig. 1A and Table 2). It is known that TNF is a major mediator of inflammation, viral replication, tumor metastasis, transplant rejection, rheumatoid arthritis and septic shock syndrome [34–36]. We speculate that the expression of TNF-α results from one of the side effects in histone H1 vaccine therapy. However, TNF was originally identified as a protein produced by the immune system that plays a major role in the suppression of tumor cell proliferation [34]. Moreover, TNF can mediate mature T cell receptor-induced apoptosis through the p75 TNF receptor [37]. We further speculate that the elevation of serum TNF-α before transplantation may be effective in inhibiting disordered (or lethal) cell proliferation (e.g., alloreactive T cell response, graft-vs.-host disease). The elevation of TNF-α in histone H1-immunized rats may contribute to prolong heart allograft survival by inhibiting alloreactive T cell response in our study. Further investigation is currently underway to elucidate the actual function of each cytokine in histone H1-immunized rats.

In summary, our present results indicate that the up-regulation of antihistone H1 Ab by histone H1 vaccination leads to the down-regulation of the alloreactive T cell response, resulting from the down-regulation of both MHC class II and CD25 molecules on splenic cells, coordination of Th1/Th2 balance, and/or the modification of serum cytokine profiles after histone H1 vaccine therapy. These results suggest that histone H1 vaccination of transplant recipients, which leads to the production of immunosuppressive factor (antihistone H1 Ab) and the modification of the
cytokine/cellular profiles, has great potential as a tolerance therapy for prospective transplantation such as living related liver transplantation. However, present vaccine protocol needs long-time immunization to get the high titer of antithistone H1 Ab. To resolve this problem, we should consider the optimum protocols (dosage and number) of vaccination for fully tolerance induction together with the application of other effective adjuvant (e.g., alum, liposome, CpG-DNA) for future clinical setting.

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