

Original articles

Transplantation

Allolymphocyte apoptosis induced by soluble liver specific antigen

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OBJECTIVE: To investigate the alloimmunogenicity of liver specific antigen and its effects on allolymphocytes.

METHODS: Liver specific antigen isolated from inbred F344 rats was used as immunogen to immunize inbred Lew rats through different immunization pathways such as low-dose long-term hind footpad, high-dose portal vein and thymus immunization. Western blotting, DNA fragments gel electrophoresis, mixed lymphocyte culture (MLC) and mixed lymphocyte hepatocyte culture (MLHC) were employed to analyze the immune state after immunization.

RESULTS: At the time point of sampling, different degree of specific low immunoresponses appeared in all immunized groups as well as cyclophosphamide (CY) treated group. Compared with group I, other groups expressed caspase-3 significantly as detected by using Western blotting. DNA fragment gel electrophoresis of splenocytes showed lymphocyte apoptosis. Compared with the group I, MLC of the experimental groups showed no significant changes except that of the group V, whereas MLHC decreased markedly ($P < 0.05$).

CONCLUSIONS: Liver specific antigen not only has alloimmunogenicity to induce alloimmunoreaction but induce antigen specific low immunoresponses and antigen specific lymphocyte apoptosis by high-dose or low-dose long-term immunization. It may be an important transplantation antigen that may lead to a novel way to liver transplantation immunotolerance.

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Key words: liver specific antigen; alloimmunoreponse; lymphocyte apoptosis; transplantation immunology

Introduction

There are specific functional cells differing from others in each tissue or organ. These cells express specific proteins to meet the demands of their specific functions. Although some proteins are universally expressed, the expression levels differ from one kind of cell to another. The discrepancy of expression levels may result in the difference of pro-

tein density and forms an antigen system called tissue specific antigen, which is independent of human leukocyte antigen (HLA), ABO antigen system and has polymorphism too.^[1,2]

It has been demonstrated that tissue specific antigens such as skin, vascular endothelial and kidney specific antigens play important roles in organ transplantation but are far from clearness. The specific antigen only expressed in liver cell membrane or liver cytoplasm and encoded by loci unlinked to the major histocompatibility (MHC) gene is called liver specific antigen (LSA). This antigen can be detected in sera of almost all liver allotransplantation recipients but its effects remain unknown yet.^[2]

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In this experiment, we tried to assess whether LSA has alloimmunogenicity to induce alloimmune-reaction and its possibility to be an important transplantation antigen using different LSA immunization pathways. The possible mechanisms of LSA and its roles in the portal vein and thymus tolerance were discussed.

Methods

Animals

Inbred male Fisher344 (RT1^o) and male Lew (RT1^l) rats were obtained from The Experimental Animal Center of Chinese Academy of Medical Sciences. The Fisher344 rats weighting 200–250 g were 10–14 weeks of age. The Lew rats were 10–14 weeks of age except those of hind footpad immunization group, 6–8 weeks of age.

Experimental protocol

Thirty Lew rats were randomly divided into five groups (6 per group). In group I or PBS-thymus or portal vein injected group, no differences were observed in the parameters between PBS-thymus and PBS-portal vein injected rats. In group II, rats were inoculated in the hind foodpad with 1 mg F344 LSA emulsified in an equal volume of Freund's complete adjuvant (100 μ l) (Sigma, Saint Lewis, USA). Additional 1 mg LSA in Freund's incomplete adjuvant (100 μ l) (Sigma, Saint Lewis, USA) was given on weeks 2, 3 and 4. In group III, 10 mg F344 LSA was immunized by portal vein injection. In group IV, 10 mg F344 LSA was immunized by thymus injection. In group V, rats were given intraperitoneally 150 mg/kg cyclophosphamide (Hengrui Corp, Jiangsu, China). All rats were sampled seven days after treatment. For all groups, the day of injection was called day 0.

Isolation of LSA

Liver specific protein S100 was prepared at 4 °C by the method described previously.^[3] Protein content was measured according to Bradford's method. Then the protein was lyophilized by CHRIST lyophilizer (B. Braun Biotech, Germany)

and stored at –80 °C.

Isolation and Western blotting of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparin-anticoagulated peripheral blood samples from all Lew rats by Ficoll-hypaque gradient centrifugation. They were washed twice with cold PBS and resuspended in lysis buffer.^[4] Equal amounts (50 μ g) of whole cellular extracts were analyzed on 10% SDS-PAGE. The protein was transferred to a nitrocellulose filter for immunoblotting. The filter was incubated with TBS-Tween 20 containing 5% nonfat milk for 2 hours and then blotted with caspase-3 (1:600) polyclonal antibody (Santa Cruz, USA) overnight at 4 °C. After washing with washing buffer, the filter was incubated with goat anti-rat IgG conjugated with horse radish peroxidase (HRP) at a concentration of 1:1000 for 2 hours. The filter was washed with washing buffer for 3 times, then added with enhanced chemiluminescent luminol reagent (Santa Cruz, USA) and exposed to X-ray film and analyzed by densitometry.

Qualitative analysis of splenocyte DNA fragmentation by gel electrophoresis

To detect whether splenocytes underwent apoptosis, Gong's method was used.^[5] Specimens containing 5 μ g of DNA were applied to 0.8% agarose gel and subjected to electrophoresis at 20 v. The gel was stained with ethidium bromide and photographed under ultraviolet illumination.

Isolation and purification of hepatocytes

F344 rat hepatocytes were isolated according to modified Seglen's perfusion technique under aseptic condition.^[6] After 60% percoll purification, small nonparenchymal cells were <1%.

Isolation of splenocytes

Spleens of F344 and Lew rats were quickly removed under aseptic condition. They were placed on sterile brass mesh over a 50 ml conical tube, and gently teased with sterile forceps. Cells were

rinsed through the brass mesh with media, and washed twice at a concentration of 300 g for 10 minutes. The viability of the cells determined by trypan blue exclusion was >90%.

Mixed lymphocyte culture and mixed lymphocyte-hepatocyte culture

Mixed lymphocyte culture (MLC) and mixed lymphocyte-hepatocyte culture (MLHC) were carried out according to the modified method of Bumgardner.^[7] To analyze MLC, spleen responder cells (5×10^5) of Lew rats were cocultured with mitomycin C (50 mg/L) (Sigma, USA) treated spleen stimulator cells (5×10^5) of F344 rats in a total volume of 200 μ l RPMI 1640 containing heat-inactivated fetal calf serum (10% v/v) in 96-well flat-bottomplates (Nuclon, Roskilde, Denmark) for 5 days. To analyze MLHC, each culture consisted of 5×10^5 responder splenocytes of Lew rat and 5000 hepatocytes of F344 rat to coculture for 5 days in 96-well flat-bottomplates. Triplicated wells were cultured for each sample at 37 °C in humidified air containing 5% CO₂. After culture for the indicated time, 10 μ l MTT solution (5 mg/ml) was added to all wells and incubated for 4 hours. 100 μ l dimethyl sulfoxide solution was added to all wells and mixed thoroughly to ensure that all crystals were dissolved. The plate was read on a microelisa reader (DYNEX MRX II, DYNEX Corp, Japan) using a test wavelength of 570 nm. The individual data were expressed as mean \pm SD of triplicate cultures.

Statistical analysis

All data were expressed as mean \pm SD and analyzed by one-way repeated measures analysis of variance (ANOVA) using SPSS software (version 11.0 for windows). A $P < 0.05$ was considered statistically significant.

Results

Caspase-3 western blotting

The increased expression level of caspase-3 in all experimental groups after LSA immunization suggested that antigen specific lymphocyte apoptosis appeared in PBMC and was the result of activation

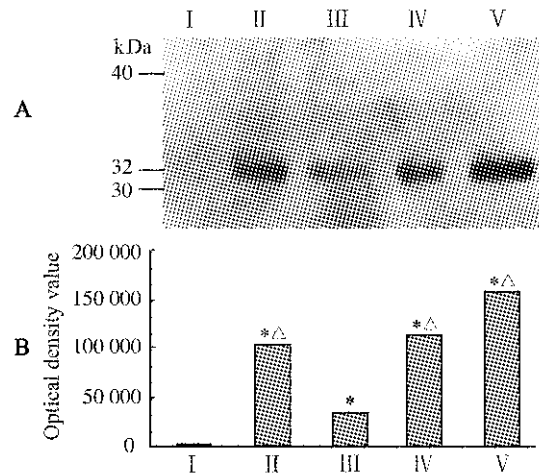


Fig. 1. PBMC caspase-3 western blotting. PBMC isolation and western blotting were described in the methods section. (A) Western blotting: I, control group; II, hind footpad immunization group; III, portal vein immunization group; IV, thymus immunization group; V, CY treated group. (B) Optical density analysis: compared with group I, * $P < 0.01$; compared with group III, $\Delta P < 0.01$.

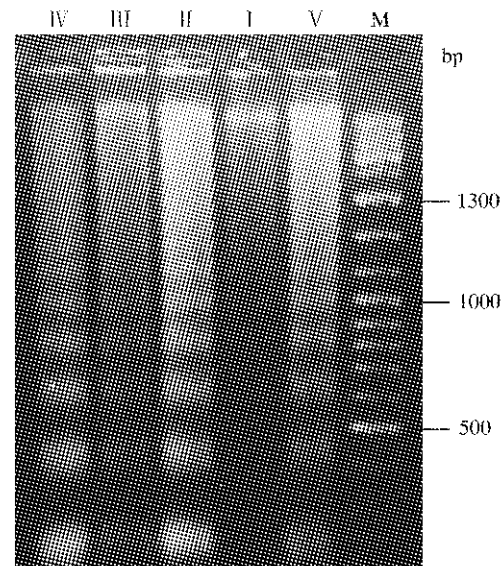


Fig. 2. Splenocyte DNA fragmentation by gel electrophoresis. DNA extraction and electrophoresis were described in the methods section. I, control group; II, hind footpad immunization group; III, portal vein immunization group; IV, thymus immunization group; V, CY intraperitoneal injection group. DNA fragmentation was observed in all but group I.

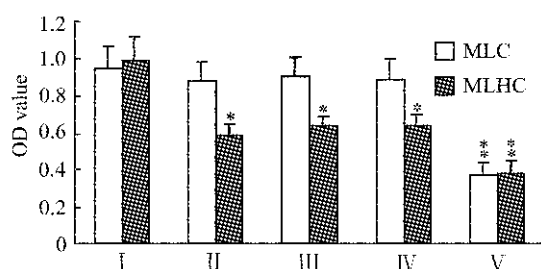


Fig. 3. Mixed lymphocyte culture and mixed lymphocyte hepatocyte culture. MLC and MLHC were described in the methods section. I, control group; II, hind footpad immunization group; III, portal vein immunization group; IV, thymus immunization group; V, CY intraperitoneal injection group. The individual data are expressed as mean \pm SD of triplicate wells and similar results were observed in two separate experiments. * $P < 0.05$; ** $P < 0.05$, control versus treated group.

of caspase-3 (Fig. 1A), and that the caspase-3 expression of group II and IV was higher than that of group III ($P < 0.05$) (Fig. 1B).

Splenocyte DNA fragmentation analysis by gel electrophoresis

DNA fragmentation of splenocytes showed DNA fragmentation in all but group I. DNA ladders was detected in group II, III and IV as well as group V, indicating the existence of apoptosis (Fig. 2).

MLC and MLHC

Compared with the control group in MLC, the OD value of the experimental groups did not decrease significantly except that of the CY treated group ($P < 0.01$) (Fig. 3). This finding showed that LSA immunization could not result in the changes of MLC, which detect reaction of lymphocytes to MHC antigens. The decreased MHC in the group V may be due to non-specific lymphocyte apoptosis, which results in decreased lymphocyte reaction to MHC and non-MHC antigens. MLHC was a parameter to show lymphocyte reaction of recipients to hepatocytes of donor. Compared with the control group, the OD value of the group II, III and IV decreased significantly 7 days after LSA immunization ($P < 0.05$), but not to the level of the

group V (Fig. 3). Treatment of Lew rats with LSA through the portal vein, thymus and hind footpad pathway abrogated the development of allospecific proliferation cells in response to hepatocytes of F344 donor. We attribute this effect to the contributions of F344 LSA that induces specific lymphocyte clone deletion.

Discussion

Although MHC-antigens have been extensively studied, the problem of rejection and/or posttransplantation hepatopathy in liver transplantation exists. Liver transplantation doesn't demand very strictly for MHC matching degree of donors and recipients, and grafted liver and recipients can survive for a long time after immunotherapy.^[8] Although multiple MHC loci especially DR matching can be seen in some cases, grafted liver loss and rejection may take place, suggesting that HLA matching has dualistic effects.^[9-10] No satisfactory explanations about the phenomenon have been made and most studies limited in MHC antigens themselves and conclusions remained controversial. It is suggested that apart from MHC antigens, other antigen systems may exist in liver transplantation immunoreaction. In this experiment, we studied the immunogenicity of LSA and non-MHC antigen by using different LSA immunization pathways.

T lymphocytes stimulated by antigens appear to be active in proliferation and function, anergic or apoptotic in complete or incomplete immunotolerance. The terminal stages of apoptosis involve the activation of a related protease family called caspase.^[11] These enzymes possess an essential cysteine residue with their active sites and cleave substrates adjacent to aspartate residues. Caspase-3 is a terminal number of caspase cascade, whose cleavage contributes to the morphological and functional changes that associated with apoptosis. Once caspase-3 activated, irreversible apoptosis takes place, showing a major difference between cell apoptosis and necrosis.^[12] In this study, the expression level of caspase-3 in all experimental groups increased after LSA immunization, suggesting that antigen specific lymphocyte apoptosis appears in

PBMC and attributes to the activation of caspase-3.

To determine the status of immune organs, splenocytes were analysed in this study. DNA ladders were detected in the group II, III, IV and V, indicating the existence of apoptosis. It is suggested that low immunoresponse induced by LSA leads to antigen specific lymphocyte apoptosis because the alloantigens phagocytized and processed by Kupffer cells after getting into the liver are present to T cells. Subsequently, antigen specific T cells are trapped and deleted because of the specific biomitabolistic environment of the liver, resulting in elimination or functional impairment of antigen specific effector T cell clone in the peripheral blood.^[13] Moreover, thymus as a specific organ has the ability to delete central clone after being modified by allogenic antigens and further to induce low immunoresponse or immunotolerance through its influence on the development and mature of T cells.^[14]

The results of MLC and MLHC after LSA immunization showed that the antigen specific lymphocytes do not lose the ability to immunoresponse to allo-MHC antigen. Compared with the control group, MLHC but not MLC in the experimental groups decreased significantly, indicating that low immunoresponses induced by LSA are antigen specific and are due to antigen specific clone deletion by analysis of caspase-3 western blotting and splenocyte DNA gel electrophoresis. Why MLHC in the group II, III and IV didn't decrease to the level of the group V may be attributable to MHC-I⁺ cells shown by hepatocytes. Because LSA is non-MHC antigen, the lymphocyte reaction induced by MHC-I⁺ hepatocytes can not be changed by LSA immunization. Noticeably, the response of recipient T cells to allografts determined by across matching of recipient T cells can not determine the response to tissue specific antigens.

We preliminarily discuss the process of recognition, presentation of LSA and its immune effects and try to explain the following phenomenon. T lymphocytes recognition process of tissue specific antigens is MHC-restricted, and includes direct and indirect recognition, the latter is the major one. In this experiment, we used protein antigen and no APC and MHC molecules of donor existed,

so the recognition of LSA was indirect. Immunologically, TCR recognizes neither MHC molecules nor antigen peptides alone. What it recognizes is surface information of MHC-antigen peptides complex. We believe that although LSA is organ specific, space conformation, electric charge qualities and distributions of MHC-antigen peptides complex are not the same because of its polymorphism, thus activating allolymphocytes.^[15] Single amino acid change in both MHC molecules and antigen peptides can influence the activation of specific lymphocyte clone.^[16] We believe that in multiple MHC loci matching, the processing ability of tissue specific antigens by recipient MHC molecules will be increased or be equal to that by donor's, thus changing indirect recognition of T lymphocytes into direct recognition. This may be one of the reasons why MHC completely matching, especially DR matching, increases the rates of graft liver dysfunction and rejection, just as what Desquenne postulated that it should make it easier to induce tolerance of skin antigen as well as other tissue specific antigen when MHC incompatibilities prevail.^[17] The data showed that MLHC but not MLC decreased significantly after LSA immunization, indicating that LSA immunization can induce low immunoresponse of recipients and that the mechanism is antigen specific lymphocyte deletion mediated by apoptosis. This is why grafted liver or recipients with high MLC can survive for a long time. Possibly, it is a split tolerance phenomenon because grafted skin may be rejected under this circumstance.^[18,19]

Interestingly, we thought the group II which may activate specific lymphocyte clone and upregulate immunoreaction also induced specific low immunoresponse at the time point of sampling. Reasonable explanation is that the long-term stimuli may result in initial lymphocyte activation, and sequentially antigen specific lymphocyte apoptosis is induced when lymphocyte contacts with the same antigen more times.^[20,21] In the group III and IV, low immunoresponse may be due to specific lymphocyte apoptosis induced by direct activation of high-dose antigen. The mechanism may coincide with the theory of high-dose/activation-associated tolerance and will be verified by further study.^[22,23]

The higher caspase-3 expression of the group

II and IV than that of the group III ($P < 0.05$) suggests that repeated low-dose long-term immunization and thymus immunization are effective pathway to induce apoptosis.

In conclusion, LSA has alloimmunogenicity to induce alloimmunoreaction. In the absence of immunosuppression or alteration of immunogenicity, transplanted purified hepatocytes or liver is unlikely to escape detection and rejection at last by recipient immune system because allogenic non-MHC antigen expressed in hepatocytes can be recognized and rejected alone. A further implication from this study is that attempts to modulate immunogenicity of whole organ allografts should be direct not only toward the allogenic MHC antigens but also toward allogenic non-MHC antigens.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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