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ISSN 1009-3095 *Journal of Zhejiang University SCIENCE* V.4, No.4, P.485-490, July-Aug., 2003

Intrathymic inoculation of donor liver specific antigen alleviates rejection of liver allotransplantation*

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Received Aug.8, 2002; revision accepted Nov.4, 2002

Abstract: Use and effects of liver specific antigen in orthotopic liver transplantations were researched in this study. Group I: syngeneic control (Wistar-to-Wistar); Group II: acute rejection (SD-to-Wistar); Group III: Thymic inoculation of SD rat LSA day 7 before transplantation. The observation of common situation and survival time, rejection grades, NF-κB activity of splenocytes and IL-2mRNA expression of grafted liver were used to analyze acute rejection severity and immune state of animals in different groups. The common situation of group I was very well after transplantation and no signs of rejection were found. Recipients of group II lost body weight progressively. All dead within day 9 to day 13 posttransplantation; median survival time was 10.7 ± 0.51 days. It was an optimal acute rejection control. As for group III, 5 out of 6 recipients survived for a long time and common situation was remarkably better than that of group II. Its rejection grades were significantly lower than that of group II ($P < 0.05$). NF-κB activity was only detected in group I at day 5 and day 7 after transplantation, whereas high activity of NF-κB was detected at all time points in groupII and the low NF-κB activity detected in group III was significantly lower than that of group II ($P < 0.05$). No IL-2mRNA expression was detected at any time point in group I, whereas high level expression was detected at all time points in group II and the low level expression only detected at day 3 in group III was significantly lower than that of group II ($P < 0.05$). Conclusion: LSA is an important transplantation antigen which is involved directly in the immunorejection of liver transplantation. We report here for the first time that intrathymic inoculation of LSA can alleviate the rejection of liver allotransplantation; and that grafts can survive for a long time thereby, thus leading to a novel way to achieve liver transplantation immunotolerance.

Key words: Liver specific antigen(LSA), Liver transplantation, Immunotolerance, NF-κB
Document code: A **CLC number:** R617

INTRODUCTION

Although MHC (major histocompatibility complex) antigens have been extensively studied, the problem of rejection in liver transplantation was not resolved completely. Liver transplantation are not strict for HLA(human leucocyte ahtigen) matching and both grafts and recipients can survive for a long time after immunotherapy. On the other hand, given HLA multiple loci matching in some cases, the likelihood of grafts dysfunction and rejection increased, which suggested that matching for HLA type may exert a dualistic effect on liver transplantation (Markus *et al.*, 1988; Doran *et al.*, 1992; Donaldson *et al.*, 1993). No satisfactory explanations have been made about it up to date; and most studies were limited to HLA antigens themselves; with the results still remaining controversial.

The specific antigens only expressed in liver cell membrane or liver cytoplasma and encoded by loci not linked to the MHC gene are called liver specific antigen(LSA). It was reported that this antigen can be detected in sera of almost all liver allotransplantation recipients but its effects remained unknown yet (Yan *et al.*, 1998). Thus in this experiment, we used donor LSA to immunize recipient rats to find the severity of rejection and the immune state of recipients posttransplantation.

* Project(NO.011106206) supported by the Science and Technology Bureau of Zhejiang Province Foundation

MATERIALS AND METHODS

Animals, reagents and instruments

Inbred male Wistar and male SD rats weighing 200 g to 250 g were purchased from the Shanghai Experimental Animal Center; [γ-^{32}P] ATP (adenosine triphosphate) (Furui Bioengineering Corp, Beijing); EMSA (electrophoresis mobility shift assay) assay kit (Promega); NF-κB double stranded oligonucleotide, 5′-AGTTGAGGGGACTTTCCC AGGC-3′, 3′-TCAACTCCCCTGAAAGGGTCCG-5′ (Santa Cruz), NF-κB double stranded mutant oligonucleotide, 5′-AGTTGAGGCGACTTTCCCA GGC-3′, 3′-TCAACTCCGCTGAAAGGGTCC G-5′ (Santa Cruz); TRIzol Reagent (Gibco BRL); MuLV (MBI Fermentas); Ultracentrifugator (Beckman); CHRIST lyophilizer (B. Braun Biotech).

Isolation of LSA

Preparation of liver specific protein S100 was carried out by the method previously described at 4℃ unless otherwise noted (Lohse *et al*., 1994). The protein content was measured according to the method of Bradford. Then the protein was lyophilized using CHRIST lyophilizer and stored at −80℃.

Surgical procedure, experimental groups and sample harvesting

Rats were anesthetized by ether inhalation. Orthotopic rat liver transplantation was performed by Kamada's two-cuff technique (Kamada *et al*., 1983). Animals surviving less than 3 days posttransplantation were attributed to technical errors and excluded from this study. Wistar rats serving as recipients were randomly divided into three groups. Group I: syngenic control (Wistar-to-Wistar); Group II: acute rejection (SD-to-Wistar). Group III: Thymic inoculation of 10 mg SD rat LSA day 7 before transplantation. All groups were subgrouped into day-1, -3, -5, -7, -12 (n = 3 each) posttransplantation respectively for sample harvesting and another subgroups (n = 6) for observation of common situation and survival time. Grafted liver specimens and splenocytes were harvest to determine morphological changes, IL-2 mRNA expression and NF-κB activity.

Histopathological examination

Grafted liver samples were fixed in 10% buffered formalin and embed in paraffin. Five micrometers think sections were affixed to slides, deparaffinized, and stained with hematoxylin and eosin to assess morphologic changes and severity of acute rejection by the Kemnitz's standard (Kemnitz *et al*., 1989).

NF-κB activity of splenocytes and EMSA specific competition inhibition

NF-κB activity detected using gel electrophoretic mobility shift assay (EMSA): Splenocytes of recipients were isolated and nuclear extracts were prepared according to the method described by Kravchenko (Kravchenko *et al*., 1995). The contents of protein were determined by the Bradford method. NF-κB oligonucleotide was end-labeled with [γ-^{32}P]ATP according to the manufacturer's recommendations. For EMSA, 10 μg of each nuclear extract was mixed with 5 × binding buffer at room temperature for 10 min. Then 1 μl [γ-^{32}P]ATP-labeled double stranded NF-κB oligonucleotide probe was added and incubated at room temperature for 20 min. The DNA/protein complex was electrophoresed on 4% nondenaturing polyacrylamide gels in 0.5 × Tris/borate/EDTA buffer to separate probe binding to NF-κB and free probe. Radioactive bands were detected by autoradiography at −70℃ followed by radiography to detect the level of retardation.

To study whether the method of EMSA was specific, Hela cell standard nuclear extract was used to bind with NF-κB protein. The specificity of binding was confirmed using 100 fold excess unlabeled NF-κB oligonucleotide as a specific competitor, 100 fold excess unlabeled unrelated oligonucleotide as a nonspecific competitor and 100 fold excess unlabeled mutant NF-κB oligonucleotide as a mutant competitor. All these unlabeled oligonucleotides were preincubated with Hela nuclear extract at room temperature for 10 min, then labeled NF-κB oligonucleotide was added. The remaining steps were the same as mentioned above.

Cytokine reverse transcription-polymerase chain reaction

Primer sequences and reaction conditions: Primers sequences used were as follows: IL-2

sense primer, 5′-GACGCTTGTCCTCCTTGTCA-3′, IL-2 antisense primer, 5′-ACCACAGTTGCTGGCTCA TC-3′(size 372bp); β-actin sense primer, 5′-TCGTACCACTGGCATTGTGA -3′, β-actin antisense primer, 5′-T CCTGCTTGCTGATCCACAT -3′ (size 645bp). Amplifications were performed at conditions: 95 ℃ for 2 minutes, 94 ℃ for 45 seconds, 56 ℃ for 45 seconds, 72 ℃ for 45 seconds, 32 cycles. The final extension step was one cycle at 72 ℃ for 10 minutes. Reaction buffer: 10 × Buffer 2.5 μl, 10 mmol/L dNTP 1 μl, MgCl 2 μl, cDNA 2 μl and 1 μl of each primer, adding ddH_2O to final volume of 25 μl.

RT-PCR: Total RNA was prepared from grafted liver with TRIzol Reagent according to the manufacturer's recommendations. For cDNA synthesis, 4 μg total RNA was reverse transcribed with MuLV reverse transcriptase according to the manufacturer's recommendations. Two microliters from the resulting cDNA solution were then amplified using specific oligonucleotides under the reaction conditions using β-actin as a "housekeeping gene" in a volume of 25 μl PCR buffer. Reaction products were run by electrophoresis on a 1.5% agarose gel for 30 min – 40 min at 100 V in 0.5 × Tris/borate/EDTA buffer, and visualized with ethidium bromide under UV light. Relative expression of cytokines were defined as optical density ratio (cytokine/β-actin) analyzed by Kodak digital science scanning system.

Statistics

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

RESULTS

Common situation posttransplantation and survival time

The common situation of group I was very good after transplantation. Recipients drank normally from day 3 posttransplantation. Normal coat recovered at day 7 with body weight increasing and all recipients survived for a long time. Recipients of group II ate badly, had tarnished coat and progressive body weight loss; all died within day 9 to day 13 posttransplantation; median survival time was 10.7 ± 0.51 days. In group III, 5 out of 6 recipients survived for a long time (one died from bile duct obstruction day 15 after transplantation); common situation resembled that of group I but remarkably better than that of group II.

Histopathological examination

No signs of rejection were found at any time point in group I with minimal portal inflammatory infiltrates. In group II, a few portal lymphocyte infiltrations combined with minimal vein endothelialitis at day 1 were found but there was no evidence of rejection. Significant portal lymphocyte infiltration with degeneration of hepatic parenchyma in some cases was found at day 3 and day 5, with average rejection grades being 1.83 and 2.67 respectively. Marked mononuclear infiltration, severe vein subendothelialitis with bridging hepatocellular necrosis can be found at both day 7 and day 12 with rejection grades of 2.87 and 3 respectively. In group III, rejection grades were 0 and 1 at day 1 and 3 respectively. Mixed but mainly lymphocytic infiltration was found both in portal tract and parenchyma without focal necrosis of the hepatic parenchyma at day 5, 7 and 12. Thus their rejection grades were all defined as 1.25 which were significantly lower than that of group II ($P < 0.05$) (Fig.1).

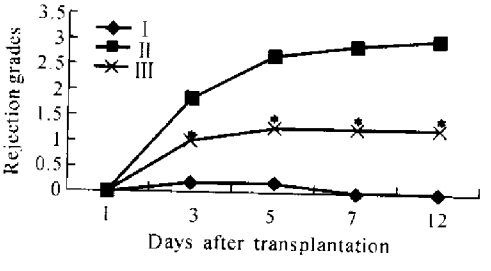

Fig.1 Rejection grades of grafted liver in different groups. Data are expressed as mean ± SD. Rejection grades of group III are significantly lower than that of group II at all but day 1 time point. Compared with group II * $P < 0.05$

EMSA specific competition inhibition and NF-κB activity of splenocytes

EMSA specific competition inhibition: No NF-κB activity was found after specific competition, whereas NF-κB activity was the same as that of positive control after nonspecific competition and mutant competition(Fig.2). These results showed that this method was specific.

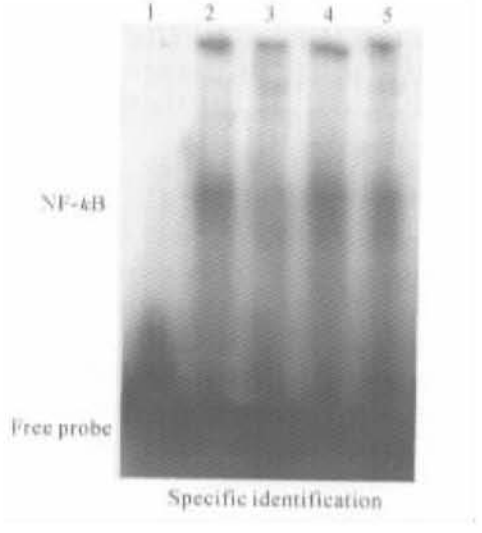

Fig.2 EMSA specific competition inhibition: Lane 1, negative control: Lane 2, positive control: Lane 3, specific competition: Lane 4, non-specific competition: Lane 5, mutant competition

NF-κB activity of splenocytes: Low NF-κB activity was only detected in group I at day 5 and day 7 after transplantation, whereas high activity of NF-κB was detected in group II at all time points and the low NF-κB activity detected in group III was significantly lower than that of group II ($P < 0.05$)(Fig.3).

Cytokine gene expression

For IL-2mRNA detection using RT-PCR in grafted liver, no expression was detected at any time point in group I, whereas high level expression was detected at all time points in group II and reached to peak at day 7 and 12 posttransplantation; the low level expression detected only at day 3 in group III was significantly lower than that of group II ($P < 0.05$). IL-2mRNA expression was consistent with histopathological damage and NF-κB activity of splenocytes.

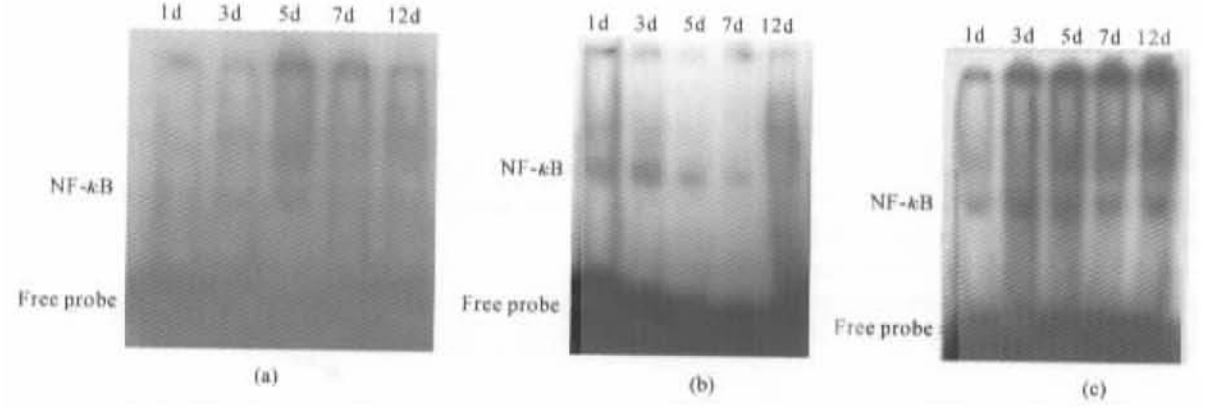

Fig.3 NF-κB activity of splenocytes at different time points posttransplantation
(a) syngeneic control (Wistar-to-Wistar); (b) acute rejection (SD-to-Wistar); (c) Thymic inoculation of LSA (SD-to-Wistar + LSA)

DISCUSSION

Allotransplantation tolerance can be induced by intrathymic inoculation of donor cellular antigen(Oluwole *et al*., 1994), and also by intrathymic inoculation of donor soluble MHC antigen (Oluwole *et al*., 1994; Stadlbauer *et al*., 2000). However, intrathymic inoculation of donor soluble non-MHC antigen have not been reported heretofore. In this study, we inoculated donor LSA into recipient thymus before liver transplantation, so rejection was greatly inhibited. The observed mild rejection of the grafted liver was significantly lower than that of the non-LSA inoculated group.

Although the rejection grades within group III increased obviously from day 3 and peaked at day 5 after transplantation, there were no significant differences between time points except for day 1($P > 0.05$). This rejection kinetic change within group III was similar to that within group I and significantly lower than that of group II at the same time point except for day 1($P < 0.05$). However, rejection of group II increased continuously, with rejection grade at day 3 posttransplantation differing significantly($P < 0.05$)

from the highest rejection grade. The rejection grades of all groups being increased rapidly within groups when compared with that of day 1 posttransplantation(Fig.1) showed that the high immunoresponses starting from day 3 to day 5 after transplantation was "rejection crisis" phase (Sharland *et al.*, 1998). That rejection grades did not increase after day 5 posttransplantation in group III, in which recipients survived for a long time suggested that the pathological damage in this group may be "self-limiting". Given longer harvesting time point, more pathological damage could probably be found.

As an important nuclear transcription factor, NF-κB is a specific sequence binding protein which can bind to promotors or enhancers of multiple genes including closely related cytokines and adhesion molecules involved in organ transplantation rejection(Patric *et al.*, 1996), thus extensively regulating the expression of these genes. Apart from that NF-κB activity change exerts crucial effects on proliferation and activation of immune cells, NF-κB is an important antiapoptotic nuclear factor(Van Antwerp *et al.*, 1996). So NF-κB activity change may play a crucial role in immunoresponses and directly influence immunoreaction posttransplantation. NF-κB regulates the transcription of IL-2 mRNA, closely related to transplantation rejection, through binding to its binding sites downstream of IL-2 gene promotor. It had been demonstrated in vitro that activated NF-κB can directly result in high level expression of IL-2 mRNA and promote T lymphocyte proliferation and activation (Kalli *et al.*, 1998). In this experiment, NF-κB activity was only detected in group I at day 5 and day 7 after transplantation, whereas high activity of NF-κB was detected at all time points in group II and the low activity of NF-κB detected in group III was significantly lower than that of group II ($P < 0.05$). This result was consistent with that of rejection severity and expression of IL-2mRNA. Thus it is believable that allotransplantation rejection is partly due to NF-κB activation at least. Intrathymic inoculation of donor LSA can inhibit NF-κB activity and further inhibit expression of IL-2mRNA and rejection posttransplantation.

TCR recognizes neither MHC molecules nor antigen peptides alone. What it recognizes is surface information on the MHC-antigen peptides complex. So we believe that although LSA is organ specific, space conformation, electric charge qualities and distributions of the MHC-antigen peptides complex are not the same due to its polymorphism; thus LSA can be recognized as nonself by allolymphocytes and further to activate this cells (Liblau *et al.*, 1996). But large numbers of activated lymphocytes may be clonally deleted due to high dose antigen immunization, which coincides with the "high-dose/activation- associated tolerance" mechanism (Bishop *et al.*, 1997). Furthermore, that the activity of antiapoptotic nuclear factor NF-κB in group III was significantly lower than that in group II also suggested the existence of lymphocyte apoptosis. In addition, thymus reeducation may also be one of the mechanisms of this tolerance(Oluwole *et al.*, 1994). T lymphocytes of recipient recognize donor LSA as self by reeducation mechanism. Besides, special attention must be paid to group III, where common situation of recipients without any transient immunosuppression was much better than that of the non-LSA treatment group and no such complication as inflammation happened. This showed that the tolerance did not result from down-regulation of the body's immunofunction; in fact, the recipients' immunuofunction was normal. This is perhaps just the optimal condition to induce donor specific tolerance and do not affect the body's immunofunction. Reasonable explanation is that strong immunoreaction to liver parenchymal cells occurs when a liver transplant is being rejected. Thus the tolerance induced by LSA to hepatocytes is donor and organ specific.

The results of this experiment showed that the thymus exerts unusual effects on tolerance induction; and that allotolerance to non-MHC antigen can be induced after thymus microenvironment contact with non-MHC antigen. It may suggest that if the immunodominant non-MHC antigen mismatch between donor and recipient could be overcome, other MHC and non-MHC antigen differences may be neglected, thus leading to long-term allograft survival across complete MHC barrier. It also suggested that MHC antigens may be not the only obstacles to successful transplantation(Lau *et al.*, 1983). The results of this study showed that LSA is involved directly in the immunorejection of liver transplantation. We reported here for the first time that intrathymic inoculation of LSA can induce permanent

and specific immunotolerance of liver allotransplantation, showed that hepatocytes are directly involved in the immunoreaction of liver transplantation. It is an important supplement to traditional theory of liver transplantation rejection which considers that rejection mainly involves in liver vascular bed, bile ducts and nonparenchymal cells, and a novel way to achieve liver transplantation immunotolerance and have far-reaching theoretical and realistic significance.

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