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The rejuvenating effects of leuprolide acetate on the aged baboon's thymus

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ABSTRACT

Background: We have previously demonstrated that the juvenile thymus plays an essential role in tolerance induced by both renal transplantation and a short course of calcineurin inhibitors. Aged thymi have a decreased ability to induce tolerance. Luteinizing hormone-releasing hormone (LHRH) is known to pharmacologically rejuvenate the thymus in rodents. In order to develop a clinically applicable regimen of transplantation tolerance in adults, we sought to determine if thymic rejuvenation would occur with LHRH agonism in non-human primates.

Methods and results: Thymic rejuvenation was evaluated by magnetic resonance imaging (MRI), histology, as well as in-vitro cellular and molecular tests. Four aged male *hamadryas* baboons underwent subcutaneous injection of a 3-month depot of Lupron (11.25 mg; LI) and were followed for 3 months. Thymi increased volumetrically by MRI. After LI, thymic cellularity markedly increased within the cortical and medullary thymus. Additionally, a significant increase in the $CD4^+/CD45RA^{hi+}$ population in the peripheral blood occurred for 50 days after LI, and flow cytometry of thymic tissue revealed a large increase in the percentage of $CD4^+/CD8^+$ cells. TREC assay corroborated enhancement in thymic function.

Conclusion: These data indicate that LI is associated with thymic rejuvenation in baboons, and further confirm that extrinsic factors play an important role in thymic rejuvenation in a non-human primate model.

1. Introduction

Tolerance remains an important goal of transplantation biology. Our laboratory has previously shown that transplantation of a class-I disparate or fully MHC-disparate kidney followed by 12-days of either high-dose CyA or FK506 facilitated allogeneic tolerance induction in MGH miniature swine [1–4]. However, surgical manipulation of the thymus or surgical thymectomy prior to transplantation interfered with tolerance induction [5,6]. Conversely, thymectomy following transplantation does not lead to tolerance abrogation [5,6]. These data suggest that tolerance induction in this model is thymus-dependent, but maintenance of tolerance is not [5–8]. Clinically, this euthymic restriction suggests that tolerance induction protocols may only be possible for children and adolescents. If the adult thymus could be rejuvenated, it would be possible to extend the potential clinical applicability of such protocols.

In small animal models, orchiectomy leading to decreased testosterone (surgical castration) has a trophic effect on the thymus [9]. By suppressing testosterone levels via chemical castration, using a 28-day course of leuprolide acetate (Lupron, LHRH agonist), investigators also observed rejuvenation of the aged rodent thymus [9]. Conversely, pregnancy (which leads to increased estrogen) has a pro-involutory effect on the thymus, and this effect is thought to modulate maternal immune responses [10]. Other laboratories have also shown that estrogen alone can induce thymic involution [11]. Although the mechanisms remain elusive, a decrease in thymic LHRH binding sites with increasing age has been correlated with thymic involution [12]. In large animal studies, we have reported that when aged thymi were transplanted into juvenile swine, these thymi were morphologically rejuvenated and restored function was observed [13]. These data suggest that (A) the immunologic environment of the juvenile animal is different from that of the adult, and (B) extrinsic factors in juvenile animals play an essential role in the rejuvenation of aged thymi. In our subsequent study performed using MGH swine model, we demonstrated that a subcutaneous injection of Lupron led to histologic thymic rejuvenation [14].

Abbreviations: CyA, Cyclosporine A; FSH, Follicle stimulating hormone; LHRH, Luteinizing hormone-releasing hormone; MGH, Massachusetts General Hospital; MHC, Major histocompatibility complex; MRI, Magnetic resonance imaging; NHP, Non-human primate; PBL, Peripheral blood lymphocyte; TREC, T cell receptor excision circle; LI, Leuprolide acetate, Lupron.

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Although miniature swine have proven to be an excellent large animal, pre-clinical model with respect to many parameters of transplantation biology [15,16], studies in non-human primates are helpful to confirm clinical applicability. Here, we attempted to rejuvenate the thymi of aged, non-human primates using Lupron. In this study we showed that the LHRH agonist rejuvenated aged, involuted thymi by 3 months using radiologic, histologic, cellular and molecular examinations.

2. Objective and hypothesis

We hypothesized that LHRH agonists might reverse the negative effects that aging has on the thymus. We investigated whether LHRH agonist injection would lead to thymic rejuvenation as assessed by imaging, as well as cellular and molecular assays in aged baboons.

3. Materials and methods

3.1. Animals

Male baboons (*Papio hamadryas*) were purchased from Mannheimer Foundation, Homestead, FL. All animals were housed at the Transplantation Biology Research Center, Massachusetts General Hospital (MGH), Boston, MA. All animal care and procedures were performed in accordance with the guidelines of the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health. The study protocol was approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

3.2. Thymic biopsy and histology

Thymic biopsies were performed through a partial sternotomy. The chest was opened, the thymus was inspected, and a wedge biopsy was obtained. Biopsy samples were read by board certified pathologists. Thymic tissues were fixed in 10% buffered formalin and embedded in paraffin for light microscopy. Tissues were stained with hematoxylin and eosin (H&E). To detect $CD3^+$ T cells, cytokeratin + thymic epithelial cells, and $FoxP3^+$ regulatory T cells in thymus, 10%-buffered, formalin fixed, and paraffin-embedded tissue sections were stained with (a) polyclonal rabbit anti-human CD3 antibody (A0452: DAKO, Glostrup, Denmark), (b) monoclonal mouse anti-human cytokeratin antibody (AE1/AE3, DAKO), and (c) polyclonal rabbit anti-human FoxP3 antibody (ab10563, Abcam) using the standard avidin–biotin–peroxidase complex technique. In order to optimize the detection of cytokeratin and FoxP3, sections were microwaved for 10 min in 0.01 M sodium citrate (pH 6.0) before incubation with the primary antibody. For CD3, tissue sections were incubated with 0.1% pepsin/0.01 N HCL for 30 min before incubation with primary antibody.

3.3. Leuprolide acetate administration

After sedation, Leuprolide acetate (Lupron, Norwood Abbey Ltd., Melbourne, Australia) 11.25 mg (a 3-month depot dose) was given as a single subcutaneous injection in the right flank.

3.4. Magnetic resonance imaging (MRI)

Thymi were imaged prior to administration of Lupron to establish baseline morphometric parameters. Additionally, animals were imaged at monthly intervals, for three months following Lupron. Animals were sedated with a continuous infusion of propofol (injection dose: at 2.5 mg/kg for the induction and at 200 ug/kg/min as maintenance) and underwent MRI. Standard T1 and T2 weighted axial and sagittal images were obtained using standard chest sequencing and 1.5 T magnet. Images were interpreted by senior radiology staff and thymic area was calculated by measuring the thymus' largest dimensions (width and length). The area (width × depth, cm^2) was calculated for comparison of images. Thymus areas per recipient body weight were compared using a single tailed Student's t-test.

3.5. Cell isolation

Peripheral blood mononuclear cells (PBMC) were obtained from blood through centrifugation of a Histopaque gradient. Absolute numbers of cells were determined by multiplying the percentage of specific cell-populations as identified on flow cytometry by the total leukocyte count measured at the same time point. Relative increases in cell counts were determined by dividing cell-population values (at a given time point) by the initial cell-population value at time zero.

3.6. Flow cytometry

T cell populations in both the peripheral blood and thymic biopsies were assessed pre- and post-LI. Key populations of interest including recent thymic emigrants (defined as $CD4^+/CD45RA^{hi}$) were followed serially in each animal following administration of Lupron.

3.7. DNA extraction

For DNA isolation from leukocytes, the buffy coat was collected and cells were washed in Hanks' buffered salt solution (Invitrogen). DNA was extracted from the PBMC using a DNeasy kit (Qiagen). The DNA concentrations were determined by nanodrop and Hoechst dye spectrophotometers. All samples were measured twice and fell within a 5% margin of error. Cells from tissues were obtained from biopsy samples. The tissue was homogenized mechanically and DNA was isolated with the DNeasy tissue isolation kit (Qiagen).

3.8. T cell receptor excision circle analysis (TREC)

For quantifying (single joint TREC) sjTREC molecules, a standard curve of sjTREC molecules was established using a cloned plasmid [17]. A known number of plasmid molecules were serially diluted 10-fold to obtain a standard curve. Standards and samples were run in triplicate, and each experiment was performed twice.

4. Results

4.1. Lupron was physiologically active in baboons

A subcutaneous injection of Lupron 3-month depot (LI) was given over the right flank in 4 aged *hamadryas* baboons in aged baboons (Table 1. Mean body weight 13.8 kg, mean age at 44.5 months old). Lupron led to a transient rise in testosterone levels, following a similar pattern to that observed following Lupron therapy in human males, indicating

Table 1
Change in area of baboon thymi by MRI presented as cm^2.

	Area of baboon thymus (cm^2) on MRI		Body weight (kg)	Area/body weight ratio (cm^2/kg)		% Increase
Animal #	Pre-LI	Post-LI		Pre-LI	Post-LI	
B299	7.36	NA	6.2	1.187	NA	NA
B267	12.81	NA	17.8	0.720	NA	NA
B285	7.80	10.80	15.3	0.510	0.705	38.46
B290	5.95	12.04	13.6	0.438	0.885	102.35
B300	9.84	18.00	13.2	0.745	1.364	82.93
B301	10.45	12.32	13.0	0.804	0.948	17.89

B299 was a juvenile control animal; B267 was an aged control animal; B285, B290, B300 and B301 were experimental animals. LI: Lupron injection.
MRIs were interpreted by an attending radiologist at Massachusetts General Hospital. Following administration of leuprolide acetate, there was a modest increase in the area of baboon thymi. Increased area (cm^2) is shown the right-most column.