

Effect of cathepsin B on thymocyte apoptosis in spontaneously hypertensive rats¹

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KEY WORDS SHR rat; thymus; cathepsin B; apoptosis; *in situ* hybridization; Northern blotting

ABSTRACT

AIM: To further investigate the mechanism of the thymus dysfunction in spontaneously hypertensive rats.

METHODS: The location of cathepsin B expression was determined by *in situ* hybridization. The expression of cathepsin B was analyzed by Northern blot, and thymocyte apoptosis was detected by TUNEL and flow cytometer, respectively. **RESULTS:** The expression of cathepsin B in thymus paralleled with thymocyte apoptosis both *in vitro* and *in vivo*. Cathepsin B transcripts were located in the plasma of thymocytes and were more over-expressed of 6- and 8-week-old SHR than those in the thymus of WKY. **CONCLUSION:** Thymocyte apoptosis in SHR is increased and this phenomenon is associated with the expression of cathepsin B.

INTRODUCTION

Spontaneously hypertensive rats (SHR) have long been established as an animal model for human essential hypertension^[1]. Besides hypertension and other cardiovascular complications, SHR also show various abnormalities of the immune system; a progressive decline of T cell functions with aging^[2,3], the appearance of natural thymocytotoxic autoantibody (NTA) beginning in the first month of age^[4], and a deficiency of thymic hormone related to T cell differentiation^[5]. Growing evidence indicates that there is a close relationship between changes in the immune system, especially the dysfunction of thy-

mus, and the development of hypertension in both SHR and humans^[6,7]. However, the molecular mechanism associated with the dysfunction of thymus in SHR is still unknown.

The thymus is one of the most important immune organs. It hosts pre-T cells and is responsible for the development and maturation of T cells. By using cDNA representational difference analysis (cDNA-RDA), combined with differential screening technique, we compared the difference in gene expression of thymus between SHR and its normotensive control Wistar Kyoto rats (WKY) and found that cathepsin B was over-expressed in the thymus of SHR^[8].

Cathepsin B is a cysteine proteinase. It was reported that cathepsin B contributed to bile-salt-induced apoptosis of rat hepatocytes^[9] and participated in the apoptosis of PC12 cells following serum deprivation^[10]. Further study indicates that cathepsin B shows caspase-processing activity. It can activate the caspase-11, and trigger the caspase-activating cascade, then lead to apoptosis of cell^[11]. Singhal^[12] *et al* reported that morphine induced apoptosis of splenocytes and enhanced the expression of cathepsin B mRNA. In our study, the over-expression of cathepsin B in thymus of SHR strongly suggests that there might be an increased thymocyte apoptosis in SHR and a relationship between cathepsin B over-expression and thymocyte apoptosis. This study was to compare the thymocyte apoptosis between SHR and WKY, and to investigate the possible connection of expression of cathepsin B with thymocyte apoptosis in thymus.

MATERIALS AND METHODS

Animals Male SHR and WKY rats (supplied by Shanghai Institute of Hypertension, Grade II, Certificate No 0237-1) were used. After sacrifice, thymus was quickly removed and rinsed in Hanks' solution at room temperature. Tissues were gently blotted and stored in liquid nitrogen for subsequent analysis as described below.

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Total RNA isolation Total RNA was prepared from SHR and WKY thymus using a guanidine thiocyanate protocol^[11]. The resulting RNA was resuspended in diethyl-pyrocyanate-treated water, quantitated by UV absorption measurements at 260 nm, and fractionated in 1 % denatured agarose gel. The absorption value ratio of OD_{260}/OD_{280} was between 1.8 and 2.0, and the ratio of 28S/18S was greater than 1.5.

Northern blot analysis For Northern blot analysis, up to 30 μg of total RNA, extracted from SHR and WKY thymus or cultured thymocytes, was subjected to 1 % denatured agarose gel electrophoresis, and capillary-transferred to Hybond N⁺ nylon membranes (Amersham, USA). The transferred RNA was linked to the nylon membranes by a UV-cross linker, then pre-hybridized in: 6 \times SSC, 2 \times Denhardt's solution, 0.1 % SDS, and salmon sperm DNA 200 mg/L at 65 $^{\circ}\text{C}$ for 3 h, and hybridized in the same condition, adding [α -³²P]dCTP-random-radiolabeled cDNA fragments which were derived from differentially expressed gene clones, and incubated overnight at 65 $^{\circ}\text{C}$. Blots were washed under stringent conditions (2 \times SSC, 0.1 % SDS, at 65 $^{\circ}\text{C}$ for 3 \times 30 min) and autoradiographed for 48 h at -80 $^{\circ}\text{C}$ with intensifying screens. Rat Glyceraldehyde-6-Phosphate Dehydrogenase (GAPDH) was used as control. The result was analyzed with Image Analysis System (HP Vectra PC).

In situ hybridization After careful fixation with 4 % polyformalin (PFA) and saturation with 30 % sucrose, thymus tissue was embedded in Optimal Cutting Temperature (OCT) compound (Miles, Elkhart, USA) for crystal section. Five- μm sections were immersed in 2 g/L glycine for 10 min at room temperature. Rinsed twice with PBS, the sections were digested with proteinase K (1 g/L) at 37 $^{\circ}\text{C}$ for 30 min, and immediately treated with 0.25 % acetic anhydride/triethanolamine 0.1 mol \cdot L⁻¹ buffer pH 8.0 at room temperature for 10 min. The sections were washed with 2 \times SSC for 2 \times 5 min and dehydrated in ethanol. The dehydrated sections were immediately subjected to hybridization with cathepsin B cDNA probes 20 $\mu\text{g}/\text{L}$ at 42 $^{\circ}\text{C}$ overnight, which were labeled with digoxin utilizing a random primer labeling system (Boehringer Mannheim, Germany) as the manufacture's procedures. After pretreatment, anti-digoxin antibody 1 mL (1:5000) was added onto the section for 2 h, and stained with NBT/BCIP solution (Boehringer Mannheim, Germany).

TUNEL assay Thymus was frozen in OCT compound (Miles, Elkhart, USA) in liquid nitrogen.

Frozen tissue sections were fixed in 2 % buffered PFA for 20 min at room temperature. After washed with PBS for 30 min, sections were treated in 0.1 % Triton X-100/0.1 % sodium citrate for 2 min at 4 $^{\circ}\text{C}$. The sections were rinsed with PBS twice, blotted, and immersed in 50 μL TUNEL reaction mixture solution [In situ cell death detection kit (Fluorescein), Boehringer Mannheim, Germany] in a humidified atmosphere at 37 $^{\circ}\text{C}$ for 1 h. The reaction was terminated by rinsing with PBS thrice and subjected to fluorescent microscopic analysis.

Cell culture Thymus was obtained from 4-week-old WKY. Thymocytes were isolated with a mesh and suspended in RPMI-1640 medium supplemented with 15 % FCS, penicillin 200 U/L, streptomycin 200 $\mu\text{g}/\text{L}$, 2-ME 50 $\mu\text{mol}/\text{L}$. The cells were adjusted to 1 \times 10¹⁰ cells/L and cultured at 37 $^{\circ}\text{C}/5\%$ CO₂ for 18 h.

Flow cytometer analysis of thymocyte apoptosis Thymocytes were fixed with 70 % ethanol. After PBS washing, they were successively treated with trypsin 30 mg/L at room temperature for 10 min, with trypsin inhibitor 50 g \cdot L⁻¹/RNase 100 mg \cdot L⁻¹ at room temperature for 10 min, and stained with propidium iodide (PI) (PI 416 mg/L, spermine 1.16 g/L) at 4 $^{\circ}\text{C}$ for 15 min. At last, the stained thymocytes were subjected to apoptotic cell count by flow cytometer.

The results were displayed as $\bar{x} \pm s$ and tested by analysis of variance.

RESULTS

The location of cathepsin B transcripts in thymus Labeled with digoxin, cathepsin B cDNA was used to probe the location of its transcripts in thymus cryosections *in situ*. The hybridization signal indicated that the transcripts of cathepsin B were located in thymocyte cytoplasm in the cortex region in SHR.

Temporal expression of cathepsin B mRNA in SHR and WKY thymus Cathepsin B was over expressed in SHR thymus, especially in 6- and 8-week-old SHR thymus (Fig 1).

Apoptosis of thymocytes The apoptotic cells were detected in thymus of 6- and 8-week-old SHR and WKY rats. The TUNEL results indicated that the number of apoptotic cells of 8-week-old SHR was greater than that of 8-week-old WKY thymus (Fig 2).

The acutely isolated thymocytes from 6-week-old SHR and WKY were subjected to apoptotic cell count by flow cytometer (FCM). The rates of apoptotic cells in

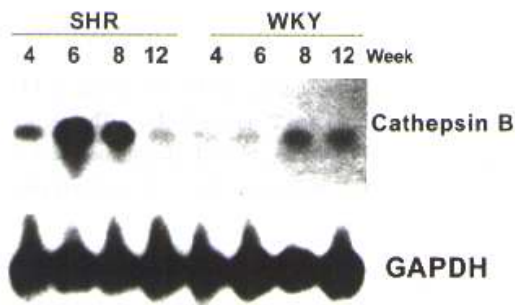


Fig 1. Northern blot analysis of cathepsin B transcripts temporally expressed in SHR and WKY thymus. Cathepsin B was over-expressed in 6-week and 8-week old SHR thymus than in WKY thymus. GAPDH was used as control to equalize the loading quantity of total RNA. $n = 4$.

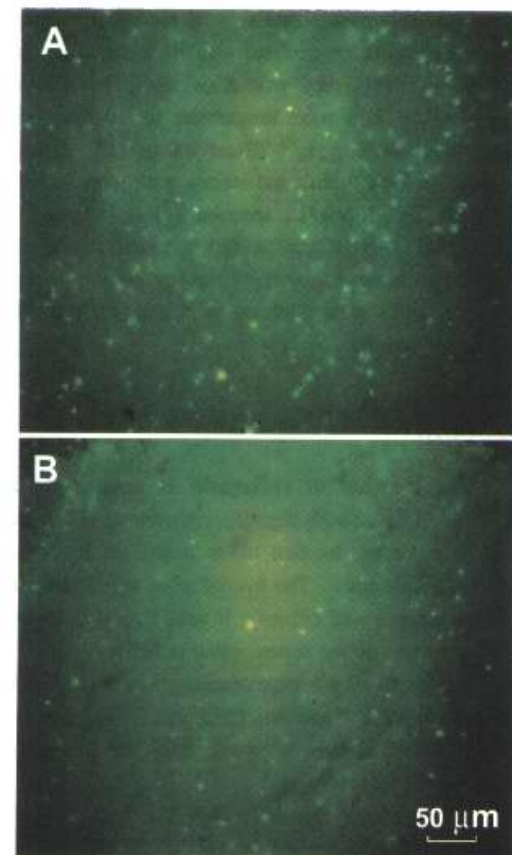


Fig 2. *In situ* detection of apoptotic cells in cryosections of thymus with TUNEL method ($\times 200$).

SHR and WKY thymocytes were 7.19 % and 4.31 %, respectively (Fig 3).

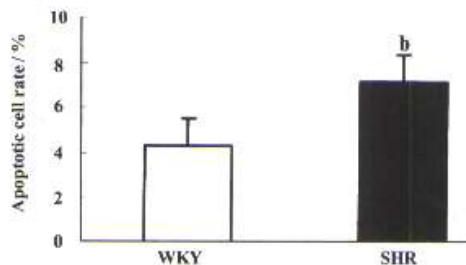


Fig 3. Flow cytometer analysis of apoptotic cells in thymus. Thymocytes were acutely isolated from 6-week-old WKY rat and SHR, subjected to propidium iodide staining. $n = 4$. $\bar{x} \pm s$. $^b P < 0.05$ vs WKY.

The expression of cathepsin B mRNA and thymocyte apoptosis induced by dexamethasone (Dex) and norepinephrine (NE) Cultured for 24 h, thymocytes from WKY rats were treated respectively with Dex 0.1 mmol/L, Dex 0.01 mmol/L, NE 0.1 mmol/L, NE 0.01 mmol/L, and PBS for 5 h, and the rates of apoptotic cells were counted by FCM and the expression of cathepsin B mRNA was analyzed by Northern blot. The rates of apoptotic cells were 23.52 % (PBS), 28.85 % (NE 0.01 mmol/L), 31.73 % (NE 0.1 mmol/L), 44.81 % (Dex 0.01 mmol/L), and 51.21 % (Dex 0.1 mmol/L) (Fig 4).

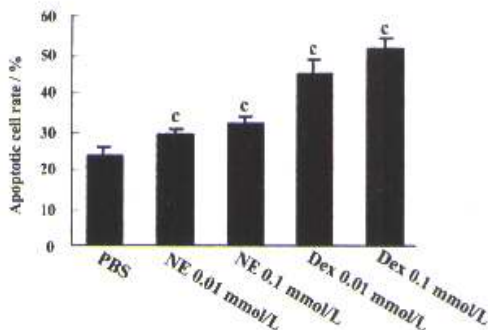


Fig 4. Histogram of thymocyte apoptosis induced by NE- and Dex-treatment. $n = 4$. $^c P < 0.01$ vs PBS group.

Similarly, the expression of cathepsin B mRNA increased in the same order shown by Northern blot analysis, ie, PBS induced the least and Dex 0.1 mmol/L induced the most expression of cathepsin B mRNA (Fig 5). But there was no correlation after statistical analysis.

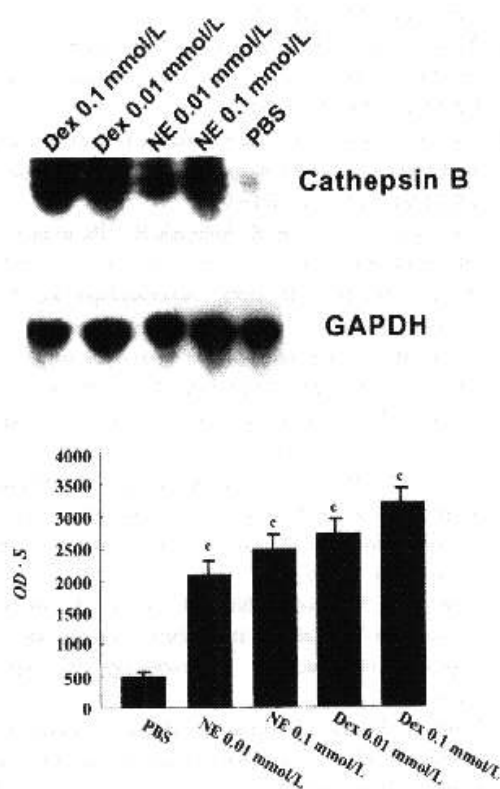


Fig 5. Northern blot analysis of the transcripts of cathepsin B in Dex- and NE-treated thymocytes. $n = 4$. $\bar{x} \pm s$. $^*P < 0.01$ vs PBS group.

DISCUSSION

Our previous study indicated that cathepsin B mRNA was over-expressed in SHR thymus. The present study showed that the expression of cathepsin B in thymus paralleled with thymocyte apoptosis both *in vivo* and *in vitro*. Cathepsin B was expressed in thymocytes and was much more expressed in thymus of 6- and 8-week-old SHR than that in WKY. Although there was no correlation in statistic, cathepsin B expression tended to be directly proportional to the number of thymocyte apoptosis. This phenomenon was again confirmed in cultured thymocytes. These maybe show the complexity of apoptotic cause or result from inadequate sample.

Apoptosis, a critical process in the immature thymocytes, requires new gene and protein synthesis. Although many factors in thymus can trigger the apoptosis of thymocytes through multiple signals, and each signal engages a unique signal-transduction pathway mediated by the products of separated genes, the final step might be

common, in which proteins or deoxynucleotides are degraded by proteases or endonucleases^[13]. Cathepsin B was recently found to be related to apoptosis of some cells. It has exo- as well as endo-peptidase activity. The endopeptidase activity of cathepsin B prefers, but is not limited to, a basic and hydrophobic amino acid at the P1 and P2 substrate sites, respectively^[14]. Such a preferred site is present in murine procaspase-11 (residues 264-IR-265) as well as in human caspase-1 (residues 295 and 296, respectively). These residues are highly conserved. An *in vitro* study^[11] proved that procaspase-11 and caspase-1 were readily processed by cathepsin B and procaspase-2, -6, -7, -14 were weak substrate, while procaspase-3 was a very poor substrate and procaspase-12 was not substrate at all for cathepsin B. Caspase-11 and caspase-1 belong to the class of initiator caspases, while caspase-3 is an executioner. Thus, given the specificity of cathepsin B for caspase-11 and caspase-1 and not for caspase-3, cathepsin B may play a regulatory role in the activation of initiator caspases rather than executioner caspases.

Dexamethasone (Dex) is a very sensitive inducer of thymocyte apoptosis^[15]. Norepinephrine (NE) can slightly increase the apoptosis of thymocytes^[16]. In this study, while different concentrations of Dex and NE induced different levels of thymocyte apoptosis, the expression of cathepsin B paralleled to the extent of apoptosis. Several other lines of research pinpoint a role for cathepsin B as an intracellular protease in active cell death or apoptosis. It has been shown that cathepsin B plays a role in apoptosis of luminal epithelial cells of the prostate and mammary gland that occurs after hormone ablation^[17]. An increased expression of cathepsin B was observed in apoptotic cells and the protein was subsequently localized in the apoptotic bodies. Moreover, an immunohistochemical study^[18] in infiltrative breast carcinomas of the natural cysteine protease inhibitor cystatin A, which inhibits cathepsin B and also cathepsin H, L, and S, suggests that the decreased apoptosis observed in tumors that express cystatin A may be due to cystatin A-mediated inhibition of cathepsin B and/or other cathepsins. Still another line of research^[19] indicated that a role of cathepsin B in 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃]-induced apoptosis. In cultured MCF7 cells, 1,25(OH)₂D₃ can up-regulate the expression of clusterin and cathepsin B, downregulate that of *bcl-2*, and exhibit increased sensitivity to apoptosis which might correlated with the presence of cathepsin B. Herein, our results provided further evidence for the association between

apoptosis and cathepsin B in thymocytes.

A pivotal process during T cell development is the clonal deletion of some sub-populations induced by apoptosis. As a result, some cells are survived, potentially autoreactive immature thymocytes are deleted, and the homeostasis of the immune system is maintained^[20].

Large numbers of precursor cells migrate into the thymus daily, where they are subjected to selection. The majority (90% - 95%) of these cells, however, die as a result of neglect (ie they are neither positively nor negatively selected). Those cells bearing TCR that recognize self major histocompatibility proteins (MHC) are positively selected. A subset of these cells recognize MHC with high affinity and are directed towards negative selection by apoptosis^[20]. Therefore, apoptosis plays a key role in the balance of immune reaction. In some cases, defects of normal cell death can lead to disease. The best example of this comes from studies of two groups of patients, each with mutations in Fas^[21, 22]. Thus, following immunization with superantigens or peptide antigens, the activated specific peripheral T lymphocytes can not be appropriately reduced or eliminated upon stimulation by apoptosis, and they continue to expand. The individuals in both groups are, for the most part, quite ill and have lymphoproliferative disorders reminiscent of those observed in *lpr/lpr* mice, named the human autoimmune lymphoproliferative syndrome. In SHR, the number of apoptotic thymocytes increased and CD8⁺ cell decreased in thymus^[23], and the number of periphery T cells also decreased^[24]. These may destroy the homeostasis of immune system, and associate with the dysfunction of the immune system in SHR.

In conclusion, we found that thymocyte apoptosis in SHR was increased, and that cathepsin B was possibly related to apoptosis in thymocytes. This work might be very helpful to interpret the dysfunction of thymus in SHR.

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组织蛋白酶 B 对自发性高血压大鼠胸腺细胞凋亡的影响¹

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关键词 近交 SHR 大鼠; 胸腺; 组织蛋白酶 B; 细胞凋亡; 原位杂交; RNA 印迹

目的: 本实验研究自发性高血压大鼠胸腺机能异常的机制。 **方法:** 用原位杂交确定蛋白酶 B 的表达位置, Northern 杂交分析组织蛋白酶 B 的表达水平, 用 TUNEL 和流式细胞仪分别检测胸腺细胞的凋亡情况。 **结果:** 在离体和在体水平, 胸腺组织蛋白酶 B 的表达与胸腺细胞凋亡伴行, 在胸腺细胞的胞浆中检测到组织蛋白酶 B 的转录产物在 6 周和 8 周的自发性高血压大鼠胸腺中的表达高于 WKY 大鼠。 **结论:** 自发性高血压大鼠的胸腺细胞凋亡增加, 与组织蛋白酶 B 的表达相关。

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