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Tyrosine phosphorylation related to thymic involution induced by diet restriction in comparison with aging

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ABSTRACT: Tyrosine phosphorylation signaling is known to be essential for the proliferation and differentiation of cells. Protein tyrosine kinases (PTKs) are present mainly in the lymphoid tissues including the thymus. The objective of the study was to investigate the expression of PTKs associated with stress-related thymic involution due to diet restriction compared with that due to aging. $p56^{lck}$ and $p59^{fyn}$ belong to the Src family membrane-associated PTKs. We found that diet-restricted rats had significantly lower thymus weights, and the expression of $p59^{fyn}$ was significantly decreased compared with the control group. In contrast, the expression of $p56^{lck}$ was not significantly different. We also found that aging-related thymic involution was not affected by the expression of those kinases. We confirmed that the mechanisms of diet-restricted thymic involution were different from those of aging-related thymic involution. It might be used as an index of chronic stress due to diet restriction in cases of child abuse or neglect.

KEYWORDS: Protein tyrosine kinases, $p59^{fyn}$, thymic involution, child abuse, diet restriction.

INTRODUCTION

The reported cases of child abuse or neglect have increased every year since the Japanese law on prevention of child abuse was legislated in 2000. Reported deaths associated with child abuse or neglect have also increased. However, it is difficult for forensic pathologists and physicians to form judgments in those cases. Previous investigators have mentioned the mechanisms of injuries and the circumstances of abused children [1, 2] after Kempe et al. first reported the 'battered-child syndrome' [3]. In the field of legal medicine, stress due to abuse or neglect was found to have led to thymic involution [4, 5] and the underlying mechanisms at the molecular level have also been reported [6, 7]. However, the difference between the mechanisms of stress-related thymic involution and those of aging-related thymic involution remains unknown.

The sensitivity of the thymus to stress is well known [8]. It has been reported that not only physically abused children but also neglected ones had shown marked involution of the thymus and that this was one of the indices of child abuse or neglect [4, 6]. Nishio and his group found that tyrosine-phosphorylated proteins had reduced remarkably in involuted thymi of stressed rats and that they could be molecular markers for thymic involution [9].

Protein tyrosine kinases (PTKs) are present mainly in lymphoid tissues including the thymus. Moreover, they are known to be essential for the proliferation and differentiation of cells. $p56^{lck}$ and $p59^{fyn}$ belong to the

Src family membrane-associated PTKs [10]. $p56^{lck}$ is principally expressed in all T lymphocytes and is involved in signal transduction for the development of T cells in the thymus and for positive selection [11-13]. $p59^{fyn}$ is universally expressed but is expressed highly in the brain and the thymus, and it has been shown to play a critical role in T-cell receptor signaling of mature thymocytes [14, 15]. Additionally, Nishio suggested that $p59^{fyn}$ is involved in fasting-induced thymic involution [16].

In the present study, we compared the mechanisms of stress-related thymic involution with those of aging-related thymic involution using anti-$p56^{lck}$ and anti-$p59^{fyn}$ antibodies.

MATERIALS AND METHODS

Chemicals and reagents

The homogenizing buffer contained 20 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 2 mM ethylenediamine-N,N,N',N'-tetra-acetic acid (EDTA), 2 mM ethylene glycol bis-N,N,N',N'-tetraacetic acid (EGTA), 2 mM sodium orthovanadate, 0.3 mg/ml benzamidine, 10 µg/ml leupeptin and 10 µg/ml aminoethyl benzenesulfonyl fluoride (AEBSF). Bicinchoninic acid protein assay reagent was purchased from Pierce, USA. Prestained Broad Range SDS-PAGE Standards for molecular weight estimation purchased from BIO-RAD, USA was used. The antibodies of clone 4G10 (1:200 dilution, Catalog # 05-321, RRID: AB_309678, Upstate Biotechnology, Inc., USA), $p56^{lck}$ (1:100 dilution, Catalog # sc-433, RRID:AB_627880, Santa Cruz Biotechnology, Inc., USA) and $p59^{fyn}$ (1:100 dilution, Catalog # sc-16, RRID:AB_631528, Santa Cruz Biotechnology, Inc., USA) were used. The HRP-conjugated secondary antibody was purchased from DAKO A/S, Denmark.

Animal study

Diet restriction model

Eight 4-week-old adolescent male Sprague-Dawley rats were used. The rats were housed in a stainless mesh cage and acclimatized to the environmental conditions for 3 days. They were divided into two groups: control rats (n=4) and stressed rats (n=4). Food (standard rat rearing pellets) and water were provided ad libitum to the control group. The stressed group had access to food every other day for two weeks with ad libitum access to water.

Aging model

Four 3-week-old, three 8-week-old and three aged male Sprague-Dawley rats were used. Each group was housed in a stainless mesh cage for 3 days as acclimation period. Food and water were provided ad libitum to all groups.

Animal ethics

All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Animal Research Laboratory, Osaka Medical College, Japan and approved by Osaka Medical College Animal Care and Use Committee (Protocol #:2019-110).

Western blot analysis

All rats were sacrificed and their thymi were excised. Immediately after the excision, they were weighed and homogenized in ten volumes of homogenizing buffer for 40 sec using a polytron homogenizer. The homogenate was centrifuged at 15,000 g for 30 min. The supernatant was collected, and protein concentrations were determined by bicinchoninic acid protein assay reagent. The same amount of protein was subjected to 10% SDS-polyacrylamide gel electrophoresis with Prestained Broad Range SDS-PAGE Standards for molecular weight estimation. They were then transferred to Polyvinylidene difluoride (PVDF) membranes (Millipore 0.45 µm) in a Bio-Rad apparatus at 100 V for 1 h. The membranes were blocked for 30 min at room temperature with 5% milk in TBS. The blots were then incubated overnight at room temperature with the mouse monoclonal anti-phosphotyrosine antibody (clone 4G10), the mouse monoclonal anti-$p56^{lck}$ antibody and the rabbit polyclonal anti-$p59^{fyn}$ antibody followed by incubation for 1 h with a secondary antibody (horseradish peroxidase-conjugated). Immunoreactive bands were visualized using a SuperSignal CL-HRP substrate system (Pierce). We replicated this experiment three times.

Statistical analysis

Statistical significance of the differences between the groups was determined by Mann-Whitney's U test. Differences were considered significant at $P<0.05$. All statistical analyses were conducted using IBM SPSS Statistics (IBM Japan, Ltd. Tokyo, Japan).

RESULTS

The effects of stress due to diet restriction

The stressed group had significantly lower body weights, lower thymus weights and thymus/body weight ratios than the control group (Figure 1). Furthermore, Western blot analysis revealed that the expression of tyrosine-phosphorylated proteins (4G10) and $p59^{fyn}$ were significantly decreased in the stress group compared with the control group, although the expression of T lymphocyte $p56^{lck}$ was not significantly different (Figure 2).

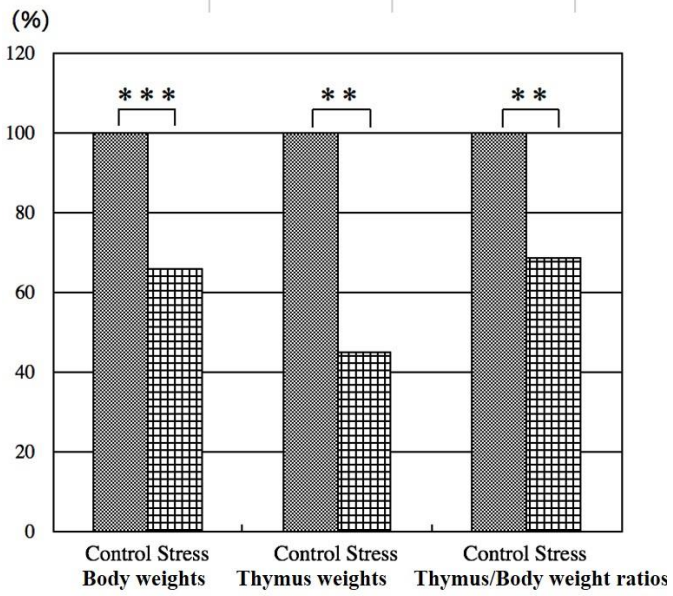

Figure 1. The differences of body weights, thymus weights and thymus/body weight ratios between the control group and the stressed group in the diet restriction model compared to each control set as 100% (***$p<0.001$, **$p<0.01$).

The decrease in expression of 4G10 reflected the decrease in overall tyrosine phosphorylation signaling, that is, stress due to diet restriction lowered the levels of overall tyrosine phosphorylation signaling in the thymi. Of tyrosine-phosphorylated proteins, $p59^{fyn}$ was significantly affected by stress due to diet restriction remarkably different from $p56^{lck}$.

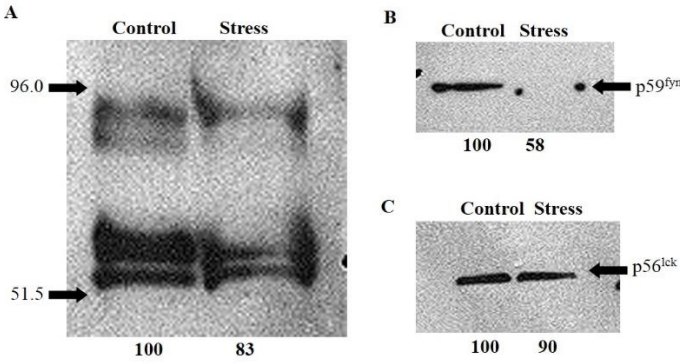

Figure 2. Representative Western blots of 4G10 (A), $p59^{fyn}$ (B) and $p56^{lck}$ (C) in the diet restriction model. Numerical digits at the bottom of each figure showed the intensity of each band compared to that of each control group set as 100 using Image J (NIH, USA).

The changes of thymus/body weight ratios in the aging model

Thymus/body weight ratios were decreased with age (Figure 3, $p<0.01$). The phenomenon called 'physiologic thymic involution (or atrophy)' was demonstrated. Usually this phenomenon is noticeable after puberty in humans followed by gradual replacement of the parenchymal tissue by fat, which leads to reduction in thymic size. This aging-related thymic involution in our rat model was not associated with the changes of expression of tyrosine-phosphorylated proteins (4G10), $p59^{fyn}$ and $p56^{lck}$ (Figure 4).

A comparison between the diet restriction model and the aging model

These results showed that the mechanisms of thymic involution due to diet restriction stress is evidently different from those by physiological changes.

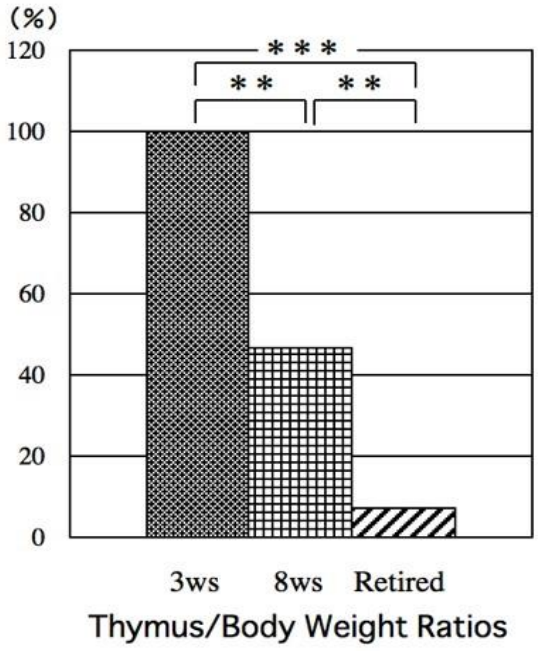

Figure 3. The changes of thymus/body weight ratios of the 3-week-old group, the 8-week-old group and the aged group in the aging model (***p<0.001, **p<0.01) demonstrated the phenomenon called 'physiologic thymic involution (or atrophy)'.

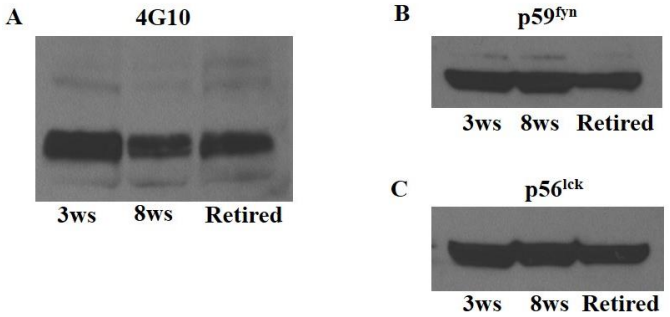

Figure 4. Representative Western blots of 4G10 (A), $p59^{fyn}$ (B) and $p56^{lck}$ (C) of the 3-week-old group, the 8-week-old group and the aged group in the aging model.

DISCUSSION

The major finding of this paper is that thymic involution induced by stress due to diet restriction is accompanied by a decrease of $p59^{fyn}$. Furthermore, our results confirmed the difference between the mechanisms of stress-related thymic involution due to diet restriction and those of aging-related thymic involution at the molecular level. The enzymatic activities of $p56^{lck}$ and $p59^{fyn}$ are regulated by protein tyrosine phosphatases, CD45. When antigens are recognized, CD45 dephosphorylates $p56^{lck}$ and $p59^{fyn}$ on tyrosine residues 505 and 528, respectively. Then, $p56^{lck}$ and $p59f^{yn}$ are autophosphorylated on tyrosine residues 394 and 417 in the catalytic kinase domain, respectively. Active $p56^{lck}$ and $p59^{fyn}$ phosphorylate the CD3ζ chain which permits association of ZAP-70 with the TCR/CD3 complex. As a result, antigen-induced signaling is transduced [10]. Although we also investigated the expression of ZAP-70 (the rabbit polyclonal anti-ZAP-70 antibody, Santa Cruz Biotechnology, USA) by Western blotting in the diet restriction model, no significant differences were detected (data not shown). This indicates that the signal transduction pathways mediated by molecules other than ZAP-70 might be involved in stress-related thymic involution.

Furthermore, the kinase activities of Src family kinases such as $p56^{lck}$ and $p59^{fyn}$ are repressed by phosphorylation of one of cytoplasmic protein tyrosine kinases, $p50^{csk}$ which is present at the highest level in lymphoid tissues [17]. We performed Western blotting analysis using the anti-$p50^{csk}$ antibody in the diet restriction model. However, a significant difference between the stressed group and the control group was not detected (data not shown).

To identify the signal transduction pathway in stress-related thymic involution, further immunological studies, including identifying the downstream targets of fyn, such as either PI(3)K or ADAP that lead to cytotoxicity or cytokine production [18], will be needed. Tanegashima et al. found that although the relative number of CD4+CD8+ double-positive thymocytes were markedly decreased and the CD4+CD8− and CD4−CD8+ subsets were relatively increased in neglected children, marked alteration of subpopulations was not observed in the physiologically involuted thymus caused by aging [7]. Moreover, in $p56^{lck}$-deficient mice, the absolute numbers of single-positive and CD4+CD8+ double-positive thymocytes were substantially reduced compared with normal littermates [10].

In our study, although the expression of $p56^{lck}$ in the stressed group was not different from the control group, the expression of $p59^{fyn}$ in the stressed group was significantly decreased. These results suggest that $p59^{fyn}$ might contribute to the alteration of subpopulations in

stress-related thymic involution. In the thymus, the capacity of leptin to reduce the rate of apoptosis and a tight connection between thymic function in malnutrition and leptin activity have been demonstrated [19, 20]. Leptin also might have played an important role in our diet restriction model.

Expression of those tyrosine-phosphorylated proteins in thymi of abused or neglected children will need to be examined. This method could contribute to the evaluation of the degree and duration of abuse or neglect. Moreover, analyzing the tyrosine- phosphorylated proteins in human peripheral T lymphocytes might assist physicians in judging whether a child has been abused or neglected for a long period.

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CONFLICTS OF INTEREST

The author declares no conflict of interest.

AUTHORS CONTRIBUTION

Izumi Takase performed the experiments, analyzed the data and wrote the manuscript.

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