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## Potential role of cytotoxic T-lymphocyte antigen 2 alpha in secretory activity of endocrine cells in mouse adenohipophysis

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### Abstract

The peptide hormones of the adenohipophysis are produced by proteolytic processing of their prohormone precursors. Cathepsin L is known to function as a major proteolytic enzyme involved in the production of the peptide hormones. The structure of the propeptide region of cathepsin L is identical to cytotoxic T-lymphocyte antigen-2 alpha (CTLA-2 $\alpha$ ) which is also shown to exhibit selective inhibitory activities against cathepsin L. However, the specific cell types synthesizing CTLA-2 $\alpha$  in mouse adenohipophysis and its functional implications as relevant *in vivo* have not been demonstrated. In this study, CTLA-2 $\alpha$  expression in the adenohipophysis was evaluated by immunohistochemistry. In both male and female mice, strong immunoreactivity was specifically detected in folliculostellate (FS) cells surrounding endocrine cells which were delineated by CTLA-2 $\alpha$ . These findings suggest that the CTLA-2 $\alpha$  may be involved in the proteolytic processing and secretion of the hormones in the adenohipophysis through regulation of cathepsin L.

**Keywords:** Adenohipophysis, CTLA-2 $\alpha$ , Immunohistochemistry, Mouse.

### Introduction

The different cell types in the adenohipophysis behave as dynamic populations. The gland has ability to adapt the absolute and relative number of each of its cell types in response to changing physiological demands. Two main cell types have been identified: the chromophobes which act as reserve cells and chromophils which are subdivided into acidophils and basophils (Melmed, 1995). The acidophils exhibit distinct cell boundaries and are further subdivided into two groups: the somatotropes which produce growth hormone and the mammatropes or lactotropes which produce luteotropic hormone or prolactin. The later initiates and maintains the secretion of milk after pregnancy (Levy, 2002). On the other hand, basophils occur in three subpopulations: the thyrotropes which secrete thyrotropic hormone for stimulating synthesis and release of thyroid hormone; the corticotropes which secrete both adenocorticotropic (ACTH) and lipotropic hormones, and the gonadotropes which produce follicle stimulating hormone and luteinizing hormone (LH) (El Etreby and El Bab, 1977; Japon *et al.*, 1994). In males, the LH is also known as interstitial cell stimulating hormone (ICSH) which stimulate production of testosterone hormone by the testicular interstitial cells. Secretory activity of the adenohipophysis is under the neuroendocrine control from the hypothalamus and is also regulated by peripheral endocrine feedback signals. Multistep proteolytic mechanisms are essential for converting prohormone precursors into active hormones (Hook, *et al.*, 1994; 2004). Prohormone processing to release the active hormone is mediated by cathepsins (Loh and Gainer, 1982; Sei *et al.*,

2003; Funkelstein *et al.*, 2008a; 2008b). In particular, cathepsin L is shown to participate in biosynthesis of ACTH,  $\beta$ -endorphin, and alpha-melanocyte stimulating hormone ( $\alpha$ -MSH) in secretory vesicles (Hwang *et al.*, 2007; Funkelstein *et al.*, 2008a). It is also involved in the conversion of proenkephalin into active enkephalin peptide neurotransmitter in chromaffin and PC12 cells (Yasothornsrikul *et al.*, 2003) and in the conversion of pro-neuropeptide Y (proNPY) into active NPY peptide in the brain where it functions as a peptide neurotransmitter and as a neuroendocrine hormone (Funkelstein *et al.*, 2008b). Under the pathological conditions, cathepsins have been observed in inflammation, tumor progression, and metastasis (Chen and Platt, 2011). The enzymatic activity of cathepsins is regulated by changes in pH and their interaction with inhibitors (Kos and Lah, 1998; Levicar *et al.*, 2002). One of the potent and specific inhibitor of cathepsin L is the cytotoxic T-lymphocyte antigen-2 $\alpha$  (CTLA-2 $\alpha$ ) which is also structurally identical to its propeptide region (Kurata *et al.*, 2003). However, the expression of CTLA-2 $\alpha$  in the adenohipophysis has not been demonstrated. The purpose of this study was to examine, by immunohistochemistry, the cellular localization and distribution pattern of CTLA-2 $\alpha$  in adenohipophysis. This information is important in understanding the mechanisms underlying secretion and regulation of peptide hormones in the gland.

### Materials and Methods

#### Tissue preparation

In this study, 10 adult mice (five males and five females) were kept in separate compartments in a small animal

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laboratory house in the controlled conditions of light (12-h light-dark cycles) and temperature (20°C–25°C). The mice were fed standard laboratory chow and water *ad libitum*. All the procedures were in compliance with the Ethical Committee of Sokoine University of Agriculture, Tanzania. The mice were anesthetized with sodium pentobarbital (60 mg/kg body weight) by intraperitoneal injection. Transcardiac perfusion with 0.01 M phosphate-buffered saline (PBS; pH 7.4) was performed followed by 4% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO) in 0.1 phosphate buffer (PB; pH 7.4). After sacrificing the mice, pituitary (hypophyses) glands were dissected, followed by fixation in 4% PFA for 2 h at 4°C. The histopathology procedure was done as described previously with minor modifications (Slaoui and Fiette, 2011). In brief, the tissues were processed in ascending ethanol series to paraffin wax. Then, tissue blocks were made and cut at 4 µm thick to produce tissue sections. Some sections were used for Hematoxylin and Eosin staining (H&E) and others for immunohistochemistry as described previously (Cuellar, 1993) using diaminobenzidine tetra-hydrochloride (DAB) for the detection of binding sites and immunofluorescence as described by Sander *et al.* (1991), with minor modifications.

#### **Routine H&E staining**

Tissue sections were deparaffinized in xylene followed by rehydration through a descending ethanol series to phosphate-buffered saline (0.01-M PBS; pH 7.4). The sections were stained using H&E in order to examine the histological organization and cellular components of the adenohypophysis.

#### **Antibody generation**

The polyclonal CTLA-2α antibody against CTLA-2α protein was obtained through affinity chromatography column with recombinant CTLA-2α conjugated resin after immunization in rabbit and antiserum preparation as previously described using recombinant protein of the CTLA-2α molecule with N-terminal poly-histidine tag (Takahashi *et al.*, 1993). The specificity of the purified antibody was characterized by Western blot as reported previously (Luziga *et al.*, 2008).

#### **Immunohistochemical examination using diaminobenzidine tetra-hydrochloride chromogen**

Deparaffinization of tissue section was done as in H&E staining. Tissue sections were immersed in a solution of 0.3% hydrogen peroxide (v/v) in distilled water for 30 min at the room temperature (RT) to inhibit endogenous peroxidase activity, followed by washing (3 × 5 min) in PBS. Sections were then incubated with 10% goat normal serum for 30 min at RT to block non-specific binding followed by incubation overnight in a dark, humid chamber at 4°C with the CTLA-2α antibody diluted at ratio of 1:500 in PBS. For negative control, PBS was applied in place of CTLA-2α antibody. Sections were then washed (3 × 5 min) in PBS followed by incubation for 30 min at RT with biotinylated goat anti-rabbit IgG (MP Biomedicals Inc., Germany).

Sections were then washed (3 × 5 min) in PBS before the incubation with streptavidin-peroxidase conjugate for 20 min at RT. The tissue section were then incubated for 3–5 min with a medium containing 0.05% 3,3'-DAB, 0.01% hydrogen peroxide, and 0.05-M Tris-HCl, pH 7.6 to visualize binding sites followed by counterstaining with hematoxylin for 30 s in order to stain the nucleus. The tissues were then rinsed for 15 min in running tap water followed by dehydration through a graded ethanol series, clearance and mounting by distyrene, plasticizer and xylene (DPX). Binding sites were assessed using Olympus BH-2 microscope fitted with Olympus camera.

#### **Immunofluorescence analysis**

To confirm the presence of CTLA-2α protein in adenohypophysis, immunofluorescence was performed on the tissue sections. The initial steps in processing tissues remained the same as for streptavidin-peroxidase method. However, the sections were incubated overnight at 4°C with CTLA-2α (1:500) IgG in PBS, pH 7.4, then washed (3 × 5 min) in PBS followed by incubation with Alexa Fluor® 488-conjugated chicken anti-rabbit IgG (fluorescein isothiocyanate (FITC)) at a dilution of 1:100 (Molecular Probes) for 1 h at RT. The tissues were then washed (3 × 5 min) in PBS and mounted. Immunolabeling was analyzed using Olympus BH-2 microscope fitted with Olympus camera.

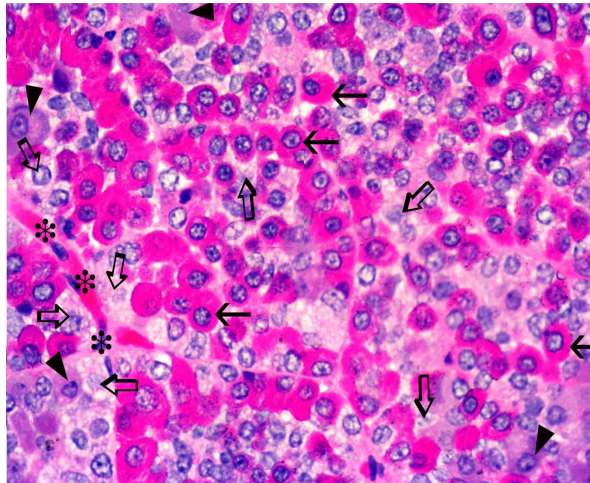
## **Results**

#### **Histological examination findings**

H&E stained sections showed that the adenohypophysis was of normal morphological organization. The gland was formed of groups of cells arranged in cords or clusters and separated by irregular blood capillaries. The cells were comprised two distinct types: chromophils and chromophobes. The chromophils are usually classified according to their shape, size, nucleus position, and the staining affinity of their granules into two major subtypes: acidophils and basophils. The acidophils generally showed distinct cell boundaries and cytoplasm was filled with specific granules that stained pink with eosin. The basophils displayed light blue nuclei and cytoplasm was filled with specific granules that stained purple with hematoxylin. The chromophobes were represented by folliculostellate (FS) cells that appeared pale in color and showed long cytoplasmic processes that surrounded adjacent endocrine cells and were in close contact with blood capillaries (Fig. 1).

#### **CTLA-2α localization in FS cells**

High levels of immunoreactivity for CTLA-2α in both male and female mice were observed in FS cells. These cells were recognized by their special tendency to extend long cytoplasmic processes to surround adjacent endocrine cells and being in close contact with capillaries. The endocrine cells were not associated with CTLA-2α expression. The endocrine cell surrounded by FS cells are thought to be somatotropes and corticotropes, which are usually located in the lateral

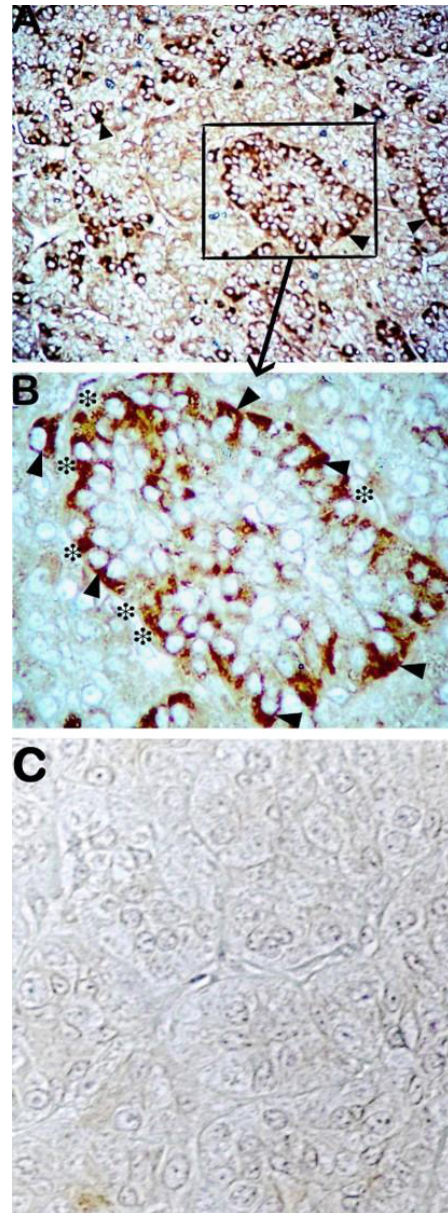


**Fig. 1.** H&E stained section demonstrating histological organization and cellular components of the hypophysis. Various types of cells are seen in the adenohypophysis. The chromophils categorized as acidophils (solid arrows) and basophils (arrow head). The acidophils show distinct cell boundaries and stain pink with eosin. The basophils display light blue nuclei and stain purple with hematoxylin. Folliculostellate (FS) cells (Open arrows) appear pale in color and extend long cytoplasmic processes to surround adjacent endocrine cells and being in close contact with blood capillaries (asterisks) (200 $\times$ ).

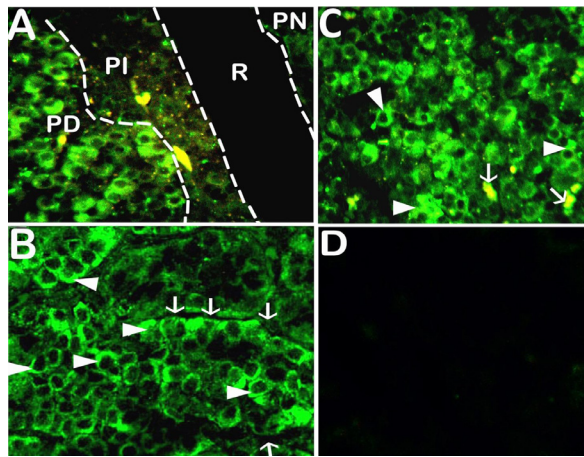
wings of cell clusters of the adenohypophysis, and also scattered in the median part. The other endocrine cells appear to be lactotropes which are usually randomly distributed throughout the adenohypophysis, mostly in the posteromedial and posterolateral aspects (Figs. 2 and 3).

### Discussion

The objective of the present study was to examine, by immunohistochemistry, the cellular localization and distribution pattern of CTLA-2 $\alpha$  in adenohypophysis and deduce its functional implications in the gland. The results showed that the CTLA-2 $\alpha$  is specifically localized at high levels in FS cells in both male and female mice. Folliculostellate cells were recognized by their special tendency to surround adjacent endocrine cells with long cytoplasmic processes. In this study, the endocrine cells surrounded by CTLA-2 $\alpha$ -reactive FS cells appeared to be the somatotropes and corticotropes. These cells are usually found in the lateral wings of cell clusters in the adenohypophysis (Melmed, 1995) and some are scattered in the median part. Others thought to be lactotropes which are usually found randomly distributed throughout the adenohypophysis, predominantly in clusters at the posteromedial and posterolateral aspects (Kovacs and Horvath, 1986). CTLA-2 $\alpha$  was not detected in all FS cells, and this could be associated with the endocrine activity of the animal and the function of individual cells at the time



**Fig. 2.** Immunohistochemical analysis of CTLA-2 $\alpha$  in the adenohypophysis using peroxidase chromogen DAB. (A) CTLA-2 $\alpha$  positive immunoreactivity in FS cells (arrowhead). The FS cells are recognized by their special tendency to surround adjacent endocrine cells with long cytoplasmic processes and being in close contact with blood capillaries (asterisks). The endocrine cells are probably the somatotropes and corticotropes, which are usually found in the lateral wings of cell clusters in the adenohypophysis and some scattered in the median part. Others are thought to be lactotropes seen randomly distributed throughout the adenohypophysis, mostly in the posteromedial and posterolateral aspects. (B) The large magnification of (A). Localization of CTLA-2 $\alpha$  in the cells is not observed in the control section (C) incubated with PBS in place of CTLA-2 $\alpha$  antibody (A: 100 $\times$ ; B and C: 400 $\times$ ).



**Fig. 3.** Immunofluorescence localization of CTLA-2 $\alpha$  in the adenohypophysis. (A) Distinct lobes of the hypophysis including the adenohypophysis which is composed of the pars distalis (PD), the pars intermedia (PI) and pars tuberalis (not shown) and the neural lobe [pars nervosa (PN)] or posterior lobe. Note also the presence of the hypophyseal cleft, the remnant of Rathke's pouch (R). High level of immunoreactivity for CTLA-2 $\alpha$  is seen in the PD and moderate in PI and absent in PN. Plates (B) and (C) Clear localization of CTLA-2 $\alpha$  in the cytoplasm of FS cells (arrow heads) that are recognized by their tendency to extend long cytoplasmic processes to surround adjacent endocrine cells and being in close contact with blood capillaries (white arrows) but is not observed in the control section. (D) Incubated with PBS in place of CTLA-2 $\alpha$  antibody (200 $\times$ ).

point when they were sampled. A normal cell responds to stimuli through coordinated changes in gene expression. The regulation of translation is often used under these circumstances because it allows immediate and selective changes in protein levels.

Several functions have been ascribed to FS cells, including phagocytosis (Stokreef *et al.*, 1986; Luziga *et al.*, 2006), stem cell function (Yoshimura *et al.*, 1977), and paracrine regulation of hormone secreting endocrine cells (Baird *et al.*, 1985). FS cells also produce many substances that influence the activity of endocrine cells, including vascular endothelial growth factor (Ferrara and Henzel, 1989), fibroblast growth factor (Ferrara *et al.*, 1987), interleukin-6 (Vankelecom *et al.*, 1993), follistatin (Kaiser *et al.*, 1992), leukemia inhibitory factor (Ferrara *et al.*, 1992), nitric oxide synthase (Ceccatelli *et al.*, 1993; Lloyd *et al.*, 1995), and leptin (Jin *et al.*, 1999). The present immunohistochemical findings on the expression of CTLA-2 $\alpha$  in FS cells expand the previous information regarding the function FS cells in adenohypophysis, indicating that FS cells are multifunctional cells and that their major function remains to be established. Indeed, previous studies show the multifunctional nature of some cells in adenohypophysis by exhibiting mixed phenotypes in which the cells may be involved

in paradoxical secretion: a secretion of a hormone evoked by a non-corresponding releasing hormone and transdifferentiation: a phenotypic switch between different mature cell types without cell division (Nunez *et al.*, 2003; Villalobos *et al.*, 2004).

It is also known that cathepsins are involved in proteolytic processing of prohormone precursors into mature hormones (Loh and Gainer, 1982; Sei *et al.*, 2003). Specifically, cathepsin L is shown to participate in various hormone processing pathways, including the biosynthesis of ACTH,  $\beta$ -endorphin, and  $\alpha$ -MSH in secretory vesicles (Funkelstein *et al.*, 2008a). The enzymatic activity of cathepsins is regulated by changes in pH and their interaction with inhibitors (Kos and Lah, 1998; Levicar *et al.*, 2002) and the CTLA-2 $\alpha$  is shown to inhibit the activity of cathepsin L with high affinity (Kurata *et al.*, 2003). Although the functional implications underlying the localization of CTLA-2 $\alpha$  in the adenohypophysis is not well established, in light of these, it is reasonable to conclude that through regulation of cathepsin L activity, CTLA-2 $\alpha$  participates in the proteolytic processing of prohormone precursors to active hormones. It is considered that FS cell synthesize CTLA-2 $\alpha$  which exert its effects on endocrine cells to which they surround with long cytoplasmic processes. In a different study, cathepsin L has been implicated in proneuropeptide processing in secretory vesicles of brain cortical neurons and in adrenal medullary chromaffin cells (Funkelstein *et al.*, 2008a). Interestingly, colocalization of cathepsin L and CTLA-2 $\alpha$  in various regions of the mouse brain has also been demonstrated by the immunofluorescence (Luziga *et al.*, 2016) indicating that the fine equilibrium between the synthesis and secretion of cathepsin L and CTLA-2 $\alpha$  is a part of the brain processes to maintain normal growth and development. Taking altogether, these results provide some clues for the possible role of CTLA-2 $\alpha$  with regard to the physiological control of hormone secretion in the adenohypophysis.

In conclusion, this study demonstrates that CTLA-2 $\alpha$  is mainly localized in FS cells in the mouse adenohypophysis. This observation is suggestive of a specialized function of CTLA-2 $\alpha$  in relation to proteolytic processing of prohormone precursors through the regulation of cathepsin activity and also opens ways to new studies on the function of CTLA-2 $\alpha$  in the endocrine system.

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#### Conflict of interest

The author declares that there is no conflict of interest.

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