Cytotoxic T-Lymphocyte Antigen-2 alpha participates in axial skeletogenesis during mouse embryo development

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SUMMARY

Cytotoxic T-lymphocyte antigen-2 alpha (CTLA-2α) has been discovered and expressed in mouse activated T-cells and mast cells. Structurally, it is homologous to the proregion of mouse cathepsin L, a lysosomal cystein proteinase. Expressed recombinant CTLA-2α is shown to exhibit selective inhibition to cathepsin L and is localized in the uterus during pregnancy where it is implicated in embryo plantation and development. CTLA-2α has also been demonstrated in the maternal side of the placenta in the deciduas basalis, metrial gland and myometrium layers in mouse but its vivo targets in the embryo are yet to be identified. We carried out studies to investigate the specific cell types synthesizing CTLA-2α protein in mouse embryo and examine its cellular localization. Immunofluorescence labeling showed intense localization of CTLA-2α in the cranium, vertebrae of cervical and thoracic region and the sternabrae. In the visceral organs, staining level was strong in the pancreas. Moderate staining was visible within the brain and remnants of the notochord. The rest of the organs including the spleen, small intestine and lungs were delineated by CTLA-2α. These findings suggest that CTLA-2α participates in an important role from the potential commitment of mesenchymal cells lineages to the ossification of axial skeleton early in embryogenesis.

Keywords: CTLA-2α, immunofluorescence, mouse embryo

INTRODUCTION

Cytotoxic T-lymphocyte antigen-2 alpha (CTLA-2α) is a distinct transcript discovered and expressed in mouse activated T-cells and mast cells (Denizot *et al*., 1989). CTLA-2α codes for 110 amino acid residues with additional N-terminal hydrophobic amino acid sequences. Structurally, it is homologous to the proregion of cysteine proteinases that are known to be involved in the cleavage of extracellular matrix and membrane proteins and thus in disease-related tissue remodeling (Cowan *et al*., 2005). CTLA-2α has been purified, characterized and expressed as a recombinant protein. It is shown to exhibit selective inhibitory activities to cathespin L-like cysteine proteinases (Kurata *et al*., 2003; Deshapriya *et al*., 2010). Two kinds of propeptide-like cysteine proteinase inhibitor proteins homologous to CTLA-2α have been discovered in organisms. The Bombyx cystein proteinase inhibitor (BCPI) identified in Bombyx mori (Yamamoto *at al*., 1999a; 1999b; Kurata *et al*., 2001) and the crammer peptide found in *Drosophila melanogaster* (Yamamoto *et al*., 2002; Comas *et al*., 2004).

Little is known regarding the cellular localization and physiological function of CTLA-2α protein in various tissues and organs in mammalian body. However, previous studies show that CTLA-2α mRNA was found to be preferentially expressed within neuronal populations in various regions of the mouse brain (Luziga *et al*., 2007). The protein was detected in neuronal dendrites and axons, suggesting its involvement in learning and memory establishment in the brain (Luziga *et al*., 2008). In the eye, CTLA-2α was shown to be produced by retinal pigmented epithelium as a releasing factor that suppresses T cell activation (Sugita *et al*., 2008). CTLA-2α was also induced in cAMP/PKA-promoted apoptosis in murine T-lymphoma cells and cardiac Fibroblasts (Zhang *et al*., 2011).

In a different study, Campo *et al*. (1996) demonstrates the expression of CTLA-2α in the uterus during pregnancy suggesting that CTLA-2α is involved in embryo plantation and development. Similar study by Bui *et al*. (2015) reveal that CTLA-2α protein is mainly localized in the maternal side of the placenta in the deciduas basalis, metrial gland and myometrium layers but was not observed in the embryonic side at different developmental stages of the mouse embryo. All these studies show that CTLA-2α performs novel physiological functions that have yet to be identified. The present study was therefore aimed at examining by immunofluorescence microscopy the distribution pattern and cellular localization of CTLA-2α in the mouse embryo in normal physiological conditions.

MATERIALS AND METHODS

Tissue preparation

All experiments conformed to the law governing the protection and control of animals (guidelines for animal experimentation) of Sokoine University of Agriculture. Ten mice in day 14 to 19 of pregnancy were used in this study. The mice were anesthetized with sodium pentobarbital (60 mg/kg) by intraperitoneal injection and transcardiacally perfused with 0.01M phosphate-buffered saline (PBS; pH 7.4), followed by 4% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO) in 0.1M phosphate buffer (PB; pH 7.4). Embryo tissues were dissected and post-fixed in 4% PFA for 2 hours at 4^{o}C before processing to paraffin wax and sectioning.

Hematoxyline and Eosin staining

Tissue blocks were cut at 4 μm thick in horizontal plane and tissue sections deparaffinized in xylene and then rehydrated through a descending ethanol series to phosphate-buffered saline (0.01M PBS-pH7.4). The sections were stained using Hematoxylin-Eosin (H&E) in order to examine the normal structures of the mouse embryo.

Antibody generation

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

Immunofluorescence analysis

Tissue sections were deparaffinized in xylene and then rehydrated through a descending ethanol series to phosphate-buffered saline (0.01M PBS-pH7.4). Sections were then immersed in a solution of 0.3 % v/v hydrogen peroxide in distilled water for 30 min at room temperature (RT) to inhibit endogenous peroxidase activity and then washed (3x5 min) in PBS. Sections were incubated with 10% goat normal serum for 30 min at RT to block non-specific binding. The sections were incubated with the CTLA-2α antibody diluted at 1:500 in PBS, for 24 h in a dark, humid chamber at 4°C. For negative control, PBS was applied in place of primary antibody. Sections were then washed (3X5min) in PBS followed by incubation with a Alexa Fluor® 488-conjugated chicken anti-rabbit IgG (FITC) at a dilution of 1:100 (Molecular Probes) for 1hour at RT. At the end of incubation, the sections were washed (3X5min) in PBS and mounted. Immunolabeling was analyzed using Olympus BH-2 microscope fitted with Olympus camera.

RESULTS

Histological findings

The histological examination of the embryo morphology was performed. The skeletal structures examined include the bones of the skull and vertebrae. Along the vertebrae the neural canal was also evident. Soft tissues structures examined include, pancreas, liver, spleen, small intestine, lungs and muscles. All these structure were in normal location and morphological appearance (Figures 1, 2 and 3).

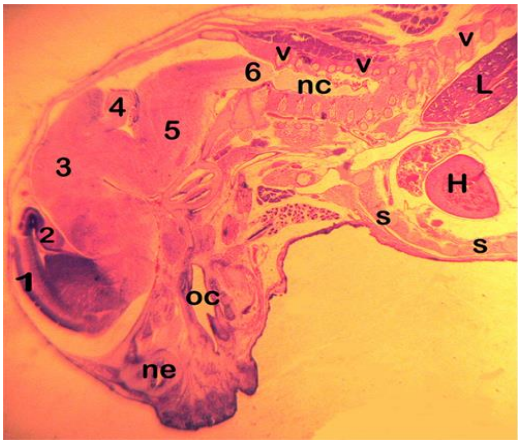

Figure 1. Hematoxyline and Eosin stained sagittal sections of mouse embryo. Cranial part of the embryo to show normal morphology of various structures. 1=Telencephalon; 2=Diencephalon; 3=Pons; 4=Cerebellum; 5=Medulla oblongata; 6=Spinal cord; nc=neural canal; v=Vertebbrae; ne=Nose epithelium; oc=Oral cavity; s= sternum; H=Heart and L=Lungs. Magnifications 100X.

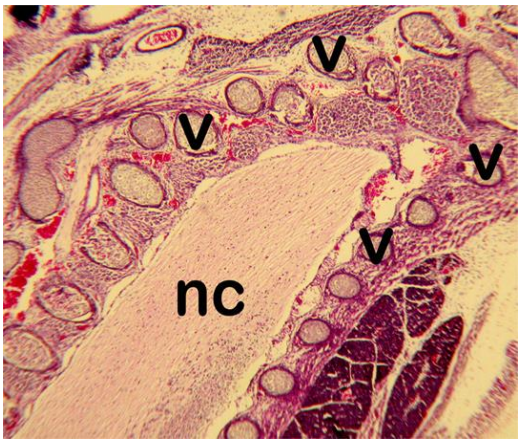

Figure 2. Hematoxyline and Eosin stained sagittal sections of mouse embryo. Normal morphological appearance of nc=Neural canal and v=Vertebrae is shown. Magnifications 200X.

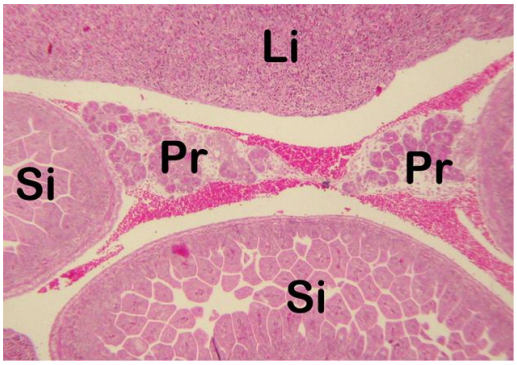

Figure 3. Hematoxyline and Eosin stained sagittal sections of mouse embryo. Normal appearance and location of visceral organs Li=Liver; Pr=Pancreas and Si=Small intestine is displayed. Magnifications 200X.

Immunofluorescence analysis

(a) Bony tissue elements

Highest level of CTLA-2α labeling was detected within developing bones of the skull or the cranium and the vertebrae. The cranium is composed of the frontal and parietal bones covering medial and lateral aspect of the brain linking the rostral capsule to the caudal cranial base. The frontal and parietal bones are formed by intramembranous bone formation in which mesenchymal cells directly differentiate into osteoblasts. Distinct labeling was observed in the bones within osteoblasts and chondrocytes (Figure 4). The vertebrae and annulus fibrosus of the intervertebral discs are derived from sclerotomal somitic mesoderm which is a derivative of paraxial mesoderm that separates into blocks of tissue, called somites. Adjacent to notochord, each somite divides into sclerotome, dermatome and myotome. The sclerotome forms the bone (vertebrae), cartilage, tendons, ligaments, annulus fibrosus of the intervertebral disc, fibrous connective tissues and meninges of the axial skeleton. Among these tissues, the vertebrae displayed distinct and intense labeling for CTLA-2α (Figure 5).

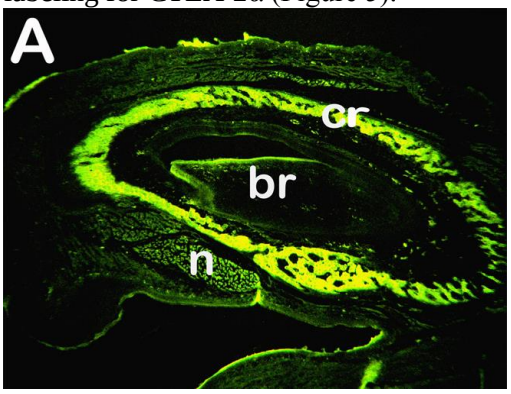

Figure 4. Immunofluorescence labeling for cytotoxic T lymphocyte antigen 2 alpha in the mouse embryo. **(A)** Entire cranium (cr) of the embryo is intensely labeled for CTLA2alpha. Moderate immunoreactivity is seen in the brain (br) and notochord (n). Magnifications 100X.

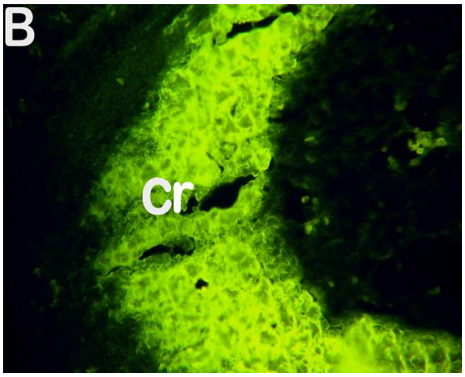

Figure 4. Immunofluorescence labeling for cytotoxic T lymphocyte antigen 2 alpha in the mouse embryo.**(B)** Higher magnification of the cranial part of the cranium (cr) or bone of the skull Magnifications 200X.

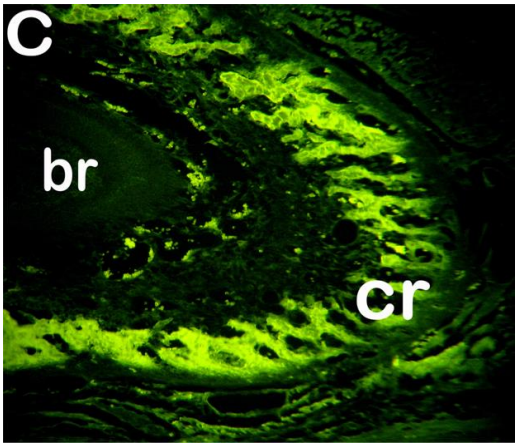

Figure 4. Immunofluorescence labeling for cytotoxic T lymphocyte antigen 2 alpha in the mouse embryo.**(C)** higher magnification of the caudal part of the cranium (cr); br=brain. Magnifications 200X.

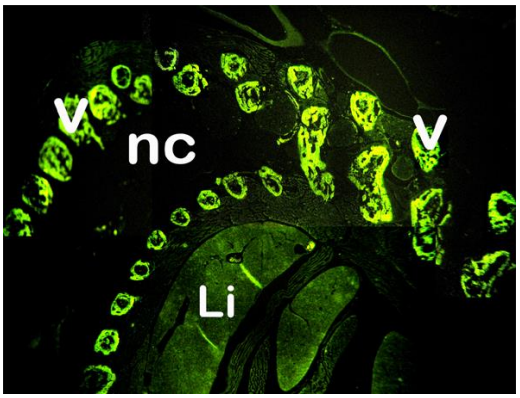

Figure 5. Immunofluorescence labeling for cytotoxic T lymphocyte antigen 2 alpha in the mouse embryo. Strong immunofluorescent signals for CTLA2alpha are observed in the vertebrae (v) but are not present in the neural canal and at lower levels in the liver (Li). Magnifications 100X.

(b) In soft tissue components

Moderate labeling for CTLA-2α was observed in the brain and remnants of the notochord which are part of the developing nervous system. Within visceral organs, positive immunoreactivity was seen in the pancreas. The liver showed weak signals but absent in the spleen, small intestines and lungs and in the control section (Figure 6).

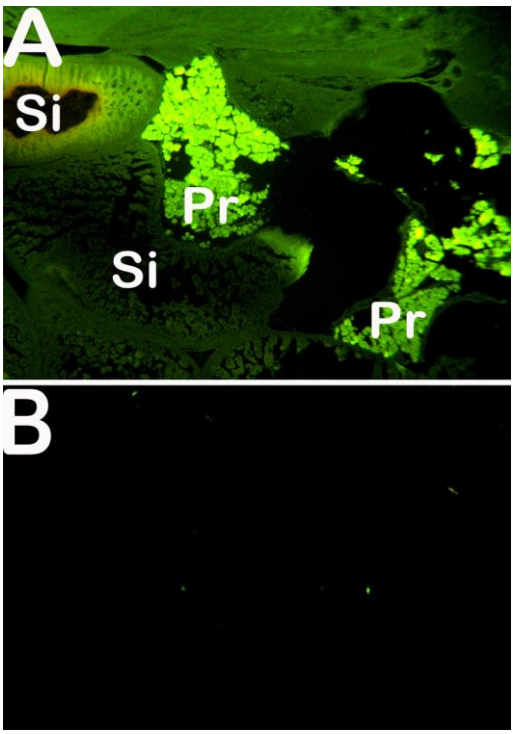

Figure 6. Immunofluorescence localization of CTLA2alpha in the viscera. **(A)** Shows strong labeling in the pancreas (Pr) but is not observed in the small intestines (Si) and in **(B)** the control section. Magnification 100X.

DISCUSSION

In the present study, we have examined the distribution pattern of CTLA-2α protein in developing mouse embryo tissues at the gestational ages ranging from 14 to 19 days. During this period CTLA-2α protein was noted in developing skeleton. Strong immunofluorescent labeling was observed in bones of developing skull and those of the vertebral column in cervical and thoracic parts. These findings indicate that CTLA-2α is involved in the processes of bone formation by regulating mesenchymal

cells that differentiate into cartilage or directly into bone. Development of bone involves many protein factors such as cbf-1 and AP1 (Wagner, 2002) and many other unknown proteins, of which CTLA-2α might be one of them.

In the vertebrate skeletal system, bone and cartilage are major tissues, which are primarily composed of three cell types: osteoblasts, chondrocytes, and osteoclasts (Bockman, 1993). In the developing embryo, osteoblasts and chondrocytes both differentiate from common mesenchymal progenitor cells but osteoclasts are of hematopoietic in origin and are brought to bones and cartilages by invading blood vessels. Two major modes of bone formation are known and both involve the transformation of a preexisting mesenchymal tissue into bone (Timothy *et al*., 2003). The intramembranous ossification in which there is direct conversion of mesenchymal tissue into bone and occurs primarily in the bones of the skull and the endochondral ossification whereby, mesenchymal cells differentiate into cartilage which is later replaced by bone. In the intramembraneous ossification, osteoblasts differentiate directly from condensed mesenchymal precursors, many of which are of the neural crest cell lineage. This process occurs mostly in the head region. In endochondral ossification, condensed mesenchymal cells give rise to both chondrocytes and osteoblasts. Chondrocytes differentiate first and form the cartilage template of the future bone while osteoblasts differentiate and mature in the periphery of the cartilage (perichondrium) to form bone collars. Chondrocyte maturation is required for endochondral ossification. This process happens in most parts of the body including the axial skeletal elements, which is formed from the somatic and lateral plate mesoderm (Bockman, 1993; Kern, 1993). Whether the localization of CTLA-2α to the cells involved in both processes of bone formation from the mesenchyme is related to its role in regulating the mesenchymal cells that differentiate into osteoblasts and chondrocytes during skull bone or cranium formation by intramembraneous ossification and to chondrocytes maturation for endochondral ossification or to a novel function is a question that remains to be resolved.

One of the macromolecules found in bones and cartilages are collagen proteins and proteoglycans. During bone formation and remodeling, cathepsins are involved in the degradation of the collagens (Kakegawa, *et al*., 1993). Cathepsin L is the major cathepsin involved in collage degradation processes in the cartilage at acidic pH. In our previous studies, we demonstrated that the structure of CTLA-2α is homologous to the proregion of cathepsins that are known to be involved in the cleavage of extracellular matrix and membrane proteins and thus in disease-related tissue remodeling (Cowan *et al*., 2005). CTLA-2α has been purified, characterized and expressed as a recombinant protein and is shown to exhibit selective inhibitory activities to cathepsin L (Kurata *et al*., 2003). Taking altogether these findings suggest that localization of CTLA-2α in axial skeleton in developing embryo participates in ossification process, or bone remodeling and resorption through regulation of cathespin L activity. However, it is not clear if CTLA-2α also degrades collagen fibers, a major and specific component of cartilage or performs specialized function during ossification.

Apart from the skeleton, intense labeling for CTLA-2α was also observed in the pancreas. The pancreas has two parts; the exocrine and endocrine components. The endocrine portions are the pancreatic islets randomly scattered throughout the organ.

Embryologically the cells of the islets and those of acini are derived from endoderm. The islet cells detach from the developing duct system and are established as endocrine cells (Bockman, 1993). Both the acini cells and the islets were intensely labeled for CTLA-2α. The acinar cell of the exocrine pancreas has the greatest rate of protein synthesis of any mammalian organ and has a highly developed endoplasmic reticulum system combined with mechanisms to modify and transport newly synthesized proteins through the secretory pathway (Case, 1978). Wartmann *et al*., 2009, showed that cathepsin L is abundantly expressed in the exocrine pancreas, sorted into the lysosomal as well as the secretory pathway of acinar cells, and secreted into pancreatic juice in human and mouse pancreas. Cathepsin L is involved in cleavage of zymogens particularly trypsnogen while cathepsin B activates the enzyme (Wartmann *et al*., 2010). Localization of CTLA-2α in the pancreas may be associated with regulation of cathepsin L in processing zymogen synthesis and activation taking into consideration that CTLA-2α is a potent and specific inhibitor of cathepsin L. In conjunction with the localization of CTLA-2α in islets where alpha and beta cells produce glucagon and insulin respectively, concerns regarding the function of CTLA-2α in processing hormone formation are also raised.

In conclusion our study demonstrates that CTLA-2α is mainly localized in axial skeleton during embryo development. This observation is suggestive of a specialized function of CTLA-2α in relation to intramembraneous and endochondral ossification as well as enzyme and hormone synthesis and secretion.

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