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Colocalization of cytotoxic T lymphocyte antigen-2 alpha and cathepsin L in ductal epithelial cells of major salivary glands of rat

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SUMMARY

Cytotoxic T Lymphocyte Antigen-2 alpha (CTLA-2 alpha) is expressed in various epithelial cells in mammalian, and is known for its specific and high affinity inhibition of cathepsin L. In the submandibular gland, cathepsin L is expressed by interstitial cells and gingival fibroblasts. However, the expression of CTLA-2 alpha in salivary glands has never been demonstrated. The objective of this study was therefore to examine the expression of CTLA-2 alpha in rat major salivary glands and to assess its cellular localization with cathepsin L, so as to deduce their functional implications in salivary secretion. Results showed that CTLA-2 alpha is strongly expressed by ductal epithelial cells in granular convoluted tubules of the submandibular salivary gland, striated and excretory ducts, blood vessels and nerve bundles of all salivary glands. Immunoreactivity was weak in intercalated ductal cells but was absent in secretory acinar cells in all the glands. Double immunofluorescence labeling was performed to determine whether CTLA-2 alpha and cathepsin L in the salivary glands were localized within the same cell. Colocalization was detected in epithelial cells of the duct system, blood vessels and nerve bundles of the glands. These results are suggestive of the specific physiological functions of CTLA-2 alpha and cathepsin L in ductal cells of the salivary glands, and implicate the two in the salivary fluid modification, processing and releasing of neuropeptides and polypeptides in the salivary fluid.

Key words: Rat – Salivary glands – CTLA-2 alpha – Cathepsin L – Immunohistochemistry

INTRODUCTION

The glandular secretory tissue of the submandibular, parotid and sublingual salivary glands is divided into lobes and lobules by connective tissue septa. Localized within the septa are blood vessels, lymphatic vessels and bundles of nerve fibers supplying the glands (Gupta and Ahuja, 2019). Unlike other salivary glands, the submandibular gland has granular convoluted tubules, which are absent in others (Mori et al., 1992). Salivary fluid is produced by secretory acinar cells as isotonic solution under stimulation of cholinergic parasympathetic nerves. Under acetylcholine and neuropeptides activation, the fluid is modified into hypotonic form as it passes along the ductal epithelium of intercalated, striated and excretory ducts towards the oral cavity (Ekström, 1999; Proctor and Carpenter, 2007).

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The involvement of acetylcholine and neuropeptides in the modification of salivary fluid is supported by the identification of sensory nerve fiber endings of neuropeptide Y, substance P and Vasoactive Intestinal Peptide (VIP) around intercalated and striated ducts, as well as blood vessels (Dunér-Engström, 1986; Ekstrom et al., 1996). The neuropeptides are implicated in cellular signaling functions, branching morphogenesis, parasympathetic innervation and epithelial proliferation (Toan et al., 2024). Ductal cells including the convoluted tubule cells, transitional cells and pillar cells are also shown to secrete growth factors, peptides and processing enzymes (Barka, 1980).

Other molecules found in ductal cells include kallikrein protease, which is involved in proteolytic processing of pentapeptide from submandibular rat 1 protein (Rougeot et al., 1994) and salivary renin that performs endocrine function in the glands (Berg et al., 1990; Menzie et al., 1974), as well as cathepsin L, which is involved in neuropeptides biosynthesis (Funkelstein et al., 2008, 2010) and is released in the salivary fluid (Mirzaii and Riahi, 2011; Gershan et al., 2015). Cathepsin L is also shown to be produced by human gingival fibroblasts during inflammation (Yamaguchi et al., 2008) and interstitial cells of submandibular glands, but not ductal cells (Sano et al., 1993).

Cytotoxic T-Lymphocyte Antigen-2 alpha (CTLA-2 alpha) is a specific and potent inhibitor of cathepsin L (Kurata et al., 2003). However, the cellular localization and physiological function of CTLA-2 alpha in major salivary glands have never been demonstrated. The hypothesis advanced was that, if CTLA-2 alpha was expressed in salivary glands, it would be an essential regulator for cathepsin L activity in the glands and control mechanism of the active concentration of neuropeptides in saliva. CTLA-2 alpha in salivary glands would also be an important link in the defense mechanisms against proteolytic damage of salivary gland tissues and ductal cells, considering the role of cathepsin L in protein degradation and remodeling of extracellular matrix (Reiser et al., 2010).

The aim of this study was therefore to examine the localization of CTLA-2 alpha in rat major salivary glands using immunohistochemistry, and to determine whether CTLA-2 alpha and cathepsin L in the salivary glands are localized within the same cell and hence implicating their interaction and relationship to salivary secretion. Localization of both CTLA-2 alpha and cathepsin L in the glands is important for better understanding of the mechanisms specifically underlying saliva secretion and regulation and its protein content and neuropeptide concentration in both health and disease.

MATERIALS AND METHODS

Animals and tissue preparation

Experiments were conducted according to the guidelines as specified by the Research Ethics Committee (DPRTC/R/186. Vol. II) of Sokoine University of Agriculture. A total of ten adult male Wistar rats aged two months, weighing 190-220 g., were used in this study.

They were kept in animal laboratory house under controlled conditions of light (12-hour light-dark cycles) and temperature (20-25°C), and fed standard laboratory chow and water ad-libitum. All rats were weighed and anesthetized by intra-muscular injection of ketamine hydrochloride (40 mg/kg) plus xylazine hydrochloride (5 mg/kg) and sacrificed. Salivary glands were dissected and fixed in boun's solution for 48 hours at room temperature. The histological procedure was done as described previously with minor modifications (Slaoui and Fiette, 2011). After fixation, the tissues were processed and embedded in paraffin wax tissue blocks followed by sectioning at 5μm thick. Some sections were used for routine Hematoxylin and Eosin (H & E) staining to evaluate the general histological organization of salivary glands and others for immunoperoxidase-DAB and immunofluorescence to evaluate localization of CTLA-2 alpha and cathepsin L in the glands.

Immunoperoxidase –DAB method

The tissue sections were deparaffinized in xylene, rehydrated through a descending ethanol series to phosphate-buffered saline (0.01M PBS-pH7.4) followed by incubation for ten min. at room temperature with hydrogen peroxide block

(Abcam, ab64261, supplied ready to use) to inhibit endogenous peroxidase activity. After washing (3x5 min.) in PBS, sections were incubated for 10 min. at room temperature with protein block (Abcam, ab64261, supplied ready to use) to block non-specific background binding. After rinsing in PBS, sections were incubated with the anti-CTLA-2 alpha antibody diluted at a ratio of 1:500 in PBS overnight in a dark, humid chamber at 4 °C. The anti-CTLA-2 alpha antibody was prepared and its specificity characterized by Western blot as reported previously (Kurata et al., 2003; Luziga et al., 2008; Nga et al., 2015; Takahashi et al., 1993). For negative controls, sections were treated as above, except that 10% goat normal serum in PBS was applied in the place of the primary antibody. Sections were then washed (3x15 min.) in PBS followed by incubation with goat anti-rabbit HRP conjugate micro-polymer (Abcam, ab64261, supplied ready to use) for 60 min. at room temperature. Sections were washed (3x15 min.) in PBS before incubation for 3-5 min. with 50X DAB chromogen solution (Abcam, ab64261, supplied ready to use) to visualize immunoreactivity. Reaction was stopped by rinsing the sections in distilled water for 5-10 min. Sections were then dehydrated through a graded ethanol series, cleared and mounted by a mixture of distyrene (a polystyrene), a plasticizer (tricresyl phosphate), and xylene (DPX). Positive immunoreactivity was evaluated using Olympus BH-2 microscope fitted with Olympus camera for image capturing. For evaluation of cathepsin L localization in the salivary glands, the same protocol was used as done in CTLA-2 alpha. Anti-cathepsin L IgY antibody was applied as primary antibody. Preparation and characterization of Anti-cathepsin L IgY antibody was reported previously (Kurata et al., 2003; Nga et al., 2015; Takahashi et al., 1993).

Immunofluorescence for CTLA-2 alpha and co-localization with cathepsin L

To confirm further the presence of CTLA-2 alpha in major salivary glands, immunofluorescence was performed on the salivary gland tissue sections. The initial steps in processing tissues remained the same as for immunoperoxidase-DAB method. However, the sections were incubated overnight at 4 °C with anti-CTLA-2 alpha antibody (1:500) IgG in PBS, pH 7.4, then washed (3X5 min.) in PBS followed by incubation with Alexa Fluor® 488-conjugated donkey anti-rabbit IgG (FITC) at a dilution of 1:100 (Molecular Probes) for one hour at room temperature. The tissues were then washed (3X15 min.) in PBS and mounted. For double labeling immunofluorescence analysis, the sections were incubated with a mixture of both anti-CTLA-2 alpha (1:500) IgG antibody and anti-cathepsin L (1:500) IgY antibody in PBS, pH 7.4 overnight in a dark, humid chamber at 4oC. For negative control, 10% goat normal serum in PBS was applied to some sections in the place of primary antibodies. Sections were then washed (3X15 min.) in PBS followed by incubation with a mixture of Alexa Fluor®488-conjugated donkey anti-rabbit IgG (FITC) and Alexa Fluor®594-conjugated goat anti-chicken IgY (TRITC) at a dilution of 1:100 (Molecular Probes, Inc. Eugene, USA) for one hour at room temperature. At the end of incubation, the sections were washed (3X15 min.) in PBS and mounted. Immunoreactivity was examined using Olympus BH-2 microscope fitted with motic camera for image capturing followed by processing using Adobe Photoshop, Version 6.0X134, BETA, 2006.

Statistical analysis

Cell count of cells showing positive immunohistochemical localization for CTLA2-alpha in major salivary glands was performed using Image J bundled with 64-bit Java 8. The cell counts were recorded in Excel software, then analyzed for statistical significance of means by two-way ANOVA using R statistical software version 4.3.1. P-value < 0.05 was considered to be significant.

RESULTS

Localization of CTLA-2 alpha in ductal epithelial cells

The secretory acinar cells of the submandibular, parotid, and sublingual salivary glands were not labelled for CTLA-2 alpha in all rats studied from the same animal tissue sections and in several immunohistochemical staining sessions using immunoperoxidase-DAB and immunofluorescence.

Weak immunoreactivity was detected in intercalated ducts of the submandibular gland but was moderate in sublingual and parotid glands. The granular convoluted tubules, which are unique to the submandibular gland, as well as striated and excretory ducts of all the glands generally showed intense distribution pattern of immunoreactivity for the CTLA-2 alpha. In granular convoluted tubules, immunoreactivity was detected within the cytoplasm of the ductal cells, which were identified by their simple columnar shape.

Immunoreactivity in striated ducts was observed within cytoplasm of the ductal cells, which were recognized by their characteristics of extensive multiple folding of the basolateral plasma membrane (Basal striations) due to the presence of a large number of mitochondria. In excretory ducts, staining was also detected in the cytoplasm of the ductal cells preferentially at basal domain (Figs. 1, 2, 3, 4). Generally, the mean cell count of cells showing positive localization for CTLA-2 alpha at 10,000 µm2 area in submandibular gland was high and significantly differed from that of parotid and sublingual glands (Fig. 7).

Localization of CTLA-2 alpha in nerve fibers, blood vessels and muscles

Morphologically, the salivary glands are covered by capsules which form septae that subdivide the salivary gland parenchyma into lobes and lobules. Blood vessels and ducts enter and leave the parenchyma through the septae. At the level of the capsule and septae are also found a large amount of white adipose tissues, as well as large and small bundles of nerve fibers. Labelling for CTLA-2 alpha was detected in the arterioles located in the septae (connective tissue sheath), specifically within the smooth muscular layer of the arterioles, but was absent in endothelial cells of the blood vessels. Nerve bundles and ducts located in the adipose tissues and skeletal muscles were also labeled for CTLA-2 alpha (Fig. 3).

Colocalization of CTLA-2 alpha and cathepsin L

To evaluate the distribution pattern of CTLA-2 alpha and cathepsin L in the salivary glands, immunohistochemistry using peroxidase DAB method was further performed on serial tissue sections using antibody against CTLA-2 alpha and cathepsin L. Results showed that CTLA-2 alpha and cathepsin L were both preferentially localized in cells of the duct system in similar distribution pattern (Fig. 5). To confirm for colocalization of CTLA-2 alpha and cathepsin L, double labeling immunofluorescence analysis was performed. The distribution pattern of CTLA-2 alpha was also found to correlate well with the distribution of cathepsin L in granular convoluted tubules of submandibular gland, striated and excretory ducts of all the glands (Fig. 6).

DISCUSSION

This study examined by immunohistochemistry the expression of CTLA-2 alpha and its concurrent localization with cathepsin L in major salivary glands of the rat. Results showed that CTLA-2 alpha and cathepsin L are localized in cells of granular convoluted tubules of the submandibular gland, striated and excretory ducts as well as blood vessels and bundles of nerve fibers but very little in intercalated ducts and none in serous and mucous secretory acinar cells of all glands. In the submandibular gland, cathepsin L is shown to be localized in interstitial cells and is involved in neuropeptides biosynthesis in secretory cells and released in the saliva (Gershan et al., 2015; Mirzaii and Riahi, 2011; Sano et al., 1993). Being a potent and specific inhibitor of cathepsin L (Kurata et al., 2003), CTLA-2 alpha was similarly thought to be localized in the acinar cells to facilitate saliva secretion. Surprisingly, both CTLA-2 alpha and cathepsin L were not detected in secretory acinar cells. This observation suggests that the two have no functional role in secretion of salivary fluid, as well as in processing of salivary contents and activation of neuropeptides needed for salivary secretion. Indeed, the serous cells produce high levels of amylase, ions and water and mucous produce viscous, enzyme-poor mucous saliva that lubricate and protect the oral mucosa (Nanci, 2013). The volume of saliva increases through parasympathetic stimulation mediated by acetylcholine of muscarinic receptors M1 and M3, whereas the sympathetic stimulation interferes with secretion of protein rich saliva through noradrenaline and b1 adrenoceptors (Baum and Wellner 1999; Proc-

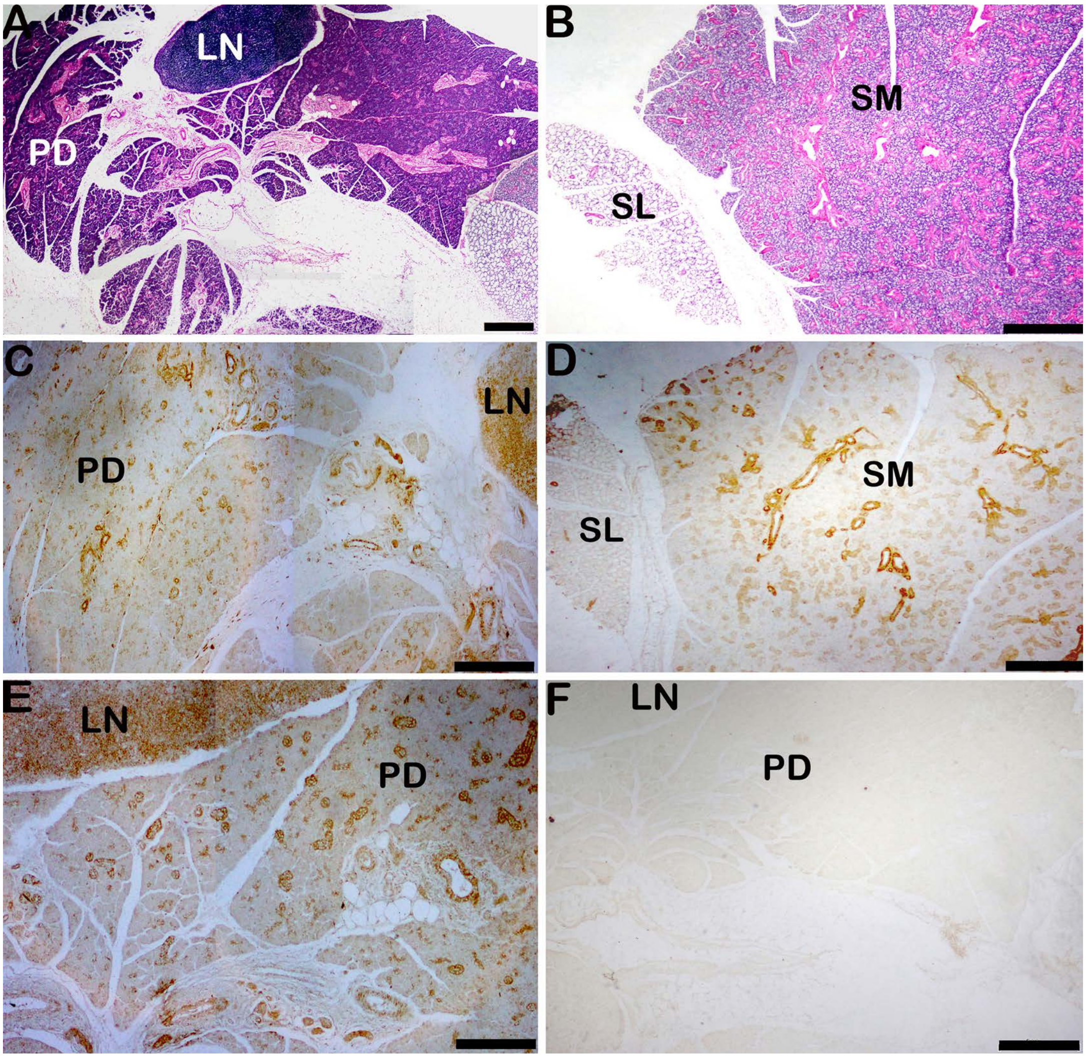

Fig. 1.- Photomicrographs of rat major salivary glands. (A) Parotid [PD] with the associated parotid lymph node [LN], (B) submandibular [SM] and sublingual [SL] salivary glands H&E-stained sections showing general overview of histological organization of the glands. (C & E) Parotid [PD] and lymph node [LN], and (D) submandibular [SM] salivary glands immunohistochemical peroxidase DAB-stained sections showing general distribution pattern of positive immunoreactivity for CTLA-2 alpha appearing as brown reaction product in ductal epithelial cells. Immunoreactivity is absent in section (F) incubated with 10% normal serum in the place of CTLA-2 alpha antibody. Scale bar A-F: 500 µm.

tor, 2016), and finally saliva transport is achieved through increase in intracellular calcium concentration (Gallacher and Smith, 1999).

Conversely, ductal cells are known for having secretory granules and synthesizing biologically active peptides such as EGF, NGF, kallikrein and renin, erythropoietin, atrial natriuretic peptides, glucagon, somatostatin, as well as other active peptides for processing proteinases and proteinase inhibitors which are secreted by alpha-adrenergic stimulants (Barka, 1980). Ductal cells are also known for having S-100 protein, neuron-specific enolase and participating in transcytosis of antibodies to the saliva from plasma cells in the surrounding connective tissue (Tandler et al., 2001). Neurons and plasma cells are one of the major cell types expressing CTLA-2 alpha and cathepsin L (Denitol et al., 1989; Luziga et al., 2016). CTLA-2 alpha in ductal cells may therefore be implicated in processing of neuropeptides and enzyme such as proteinases which are secreted by alpha-adrenergic stimulants of sympathetic nerve. Although the relationship between neuropeptides and CTLA-2 alpha has never been demonstrated, however, colocalization of CTLA-2 alpha and cathepsin L in various biological tissues including brain (Luziga, et al., 2016) and pancreas (Luziga, 2020) is suggestive of CTLA-2 alpha roles in proteolytic processing of neuropeptides through regulation of cathepsin L activity and providing defense mechanisms against proteolyt-

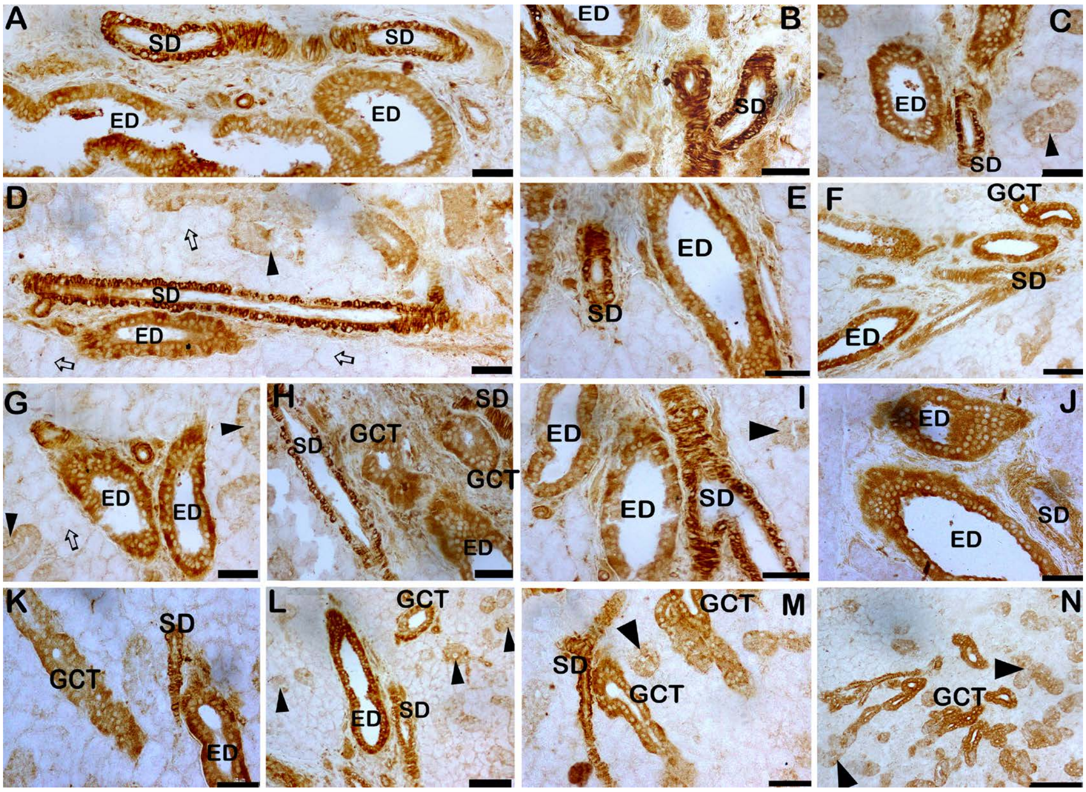

Fig. 2.- Immunohistochemical images of rat submandibular salivary glands. (A-N) Higher magnification images showing strong immunoreactivity for CTLA-2 alpha in submandibular gland. Note: strong immunoreactivity appears in epithelial cells of excretory ducts (ED) with large lumen, striated ducts (SD) characterized by extensive multiple folding of the basolateral plasma membrane (due to the presence of a large number of mitochondria) and granular convoluted tubules (GCT) characterized by columnar shaped cells. Faint immunoreactivity seen in epithelial cells of intercalated ducts (solid arrowhead) that exhibit low cuboidal shape with centrally located nuclei but the acini (open arrows) are not labelled for CTLA-2 alpha. Scale bar A-J: 50 µm; K-M: 100 µm; N: 200 µm.

ic damage of cathepsin L to the tissues of the duct system.

Cathepsin L is also reported to occur in salivary fluid (Mirzaii and Riahi, 2011), produced in human gingival fibroblasts and its production is enhanced under the influence of interleukin-6, implicating inflammatory processes in its expression (Yamaguchi et al., 2008). Sano et al. (1993) also reported on the localization of cathepsin L in mouse salivary glands, an observation that coincides well with the findings of this study. The only exception to this is that Sano et al. (1993) found cathepsin L in interstitial cells (macrophages), but not in ductal cells, as observed in this study.

The secretory activity of salivary glands is controlled by the autonomic nervous system through neuropeptides such as neuropeptide Y, VIP and substance P, which are localized in sensory nerve fiber endings. The parasympathetic nerve innervates acinar cells with a major population of neuropeptide Y containing nerve fibers, while the sympathetic nerve innervates intercalated and striated ducts, as well as blood vessels (Dunér-Engström, 1986; Ekstrom et al., 1996). Studies on denervation activities of nerve fibers show that the intensity of neuropeptide Y immunoreactive nerve fibers close to acinar cells is remarkably reduced following parasympathetic denervation, whereas sympathetic denervation significantly reduced the number of neuropeptide Y immunoreactive nerve fibers close to ducts and blood vessels (Ekstrom et al., 1996; Schultz et al., 1994). In this study, the ducts and blood vessels were labeled for CTLA-2 alpha and cathepsin L, but not the acini. This observation supplements previous suggestions that CTLA-2 alpha and cathepsin L participate in regulatory function of neurotransmitters in sympathetic nerve fibers, which supply the ducts and blood vessels, but not the parasympathetic nerve fibers, which supply the acini. However, further investigation is re-

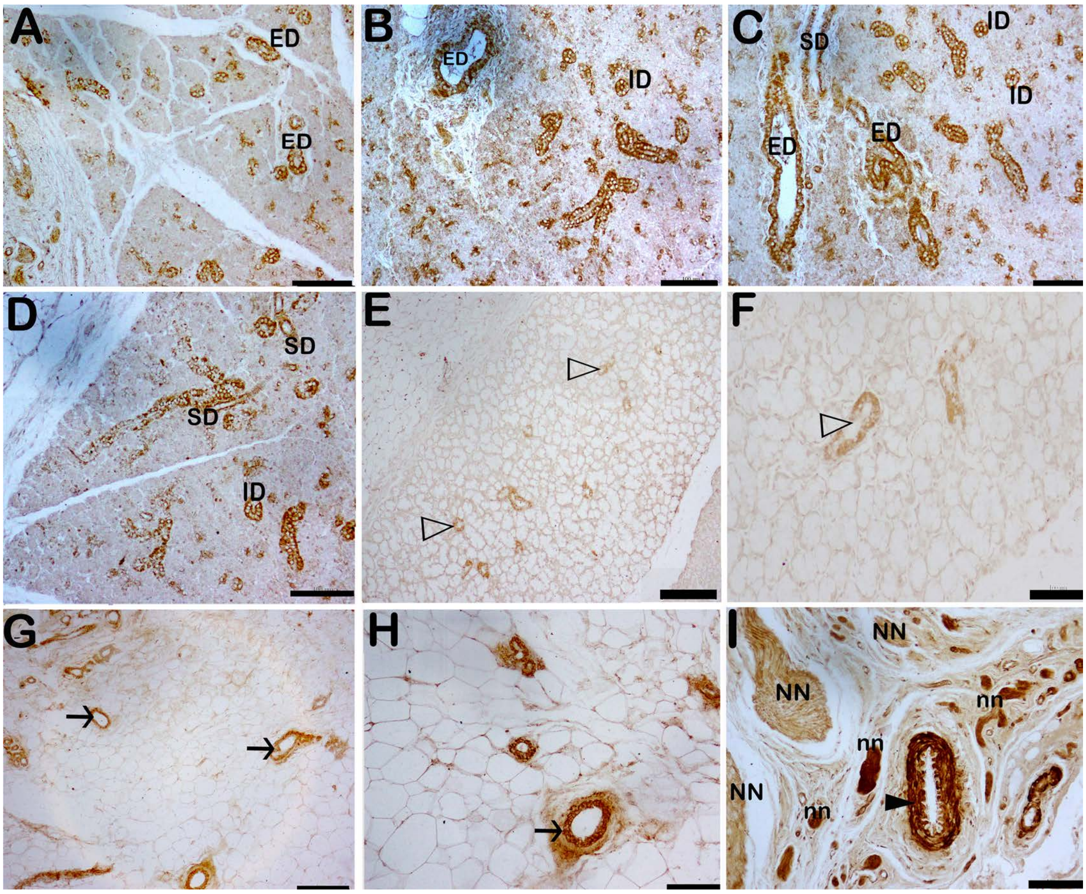

Fig. 3.- Photomicrographs of CTLA-2 alpha immunostaining in parotid, sublingual and associated structures. (A-D): Parotid gland showing positive immunoreactivity for CTLA-2 alpha in epithelial cells lining the ducts. Note: strong brown reaction product in the epithelial cells of excretory ducts (ED), striated ducts (SD) and intercalated ducts (ID). (E-F): Sublingual gland showing moderate immunostaining in the ductal cells (open arrowheads). (G-H): Adipose tissue showing moderate to strong immunoreactivity in the ductal cells and (I) shows strong immunostaining in interlobular artery in smooth muscles of tunica media (solid arrowhead) but not observed in tunica intima, strong in small (nn) but moderate in large (NN) nerve bundles located in the interstitial glandular stroma. Scale bar A-D & F-H: 100 µm; E: 200 µm; I: 50 µm.

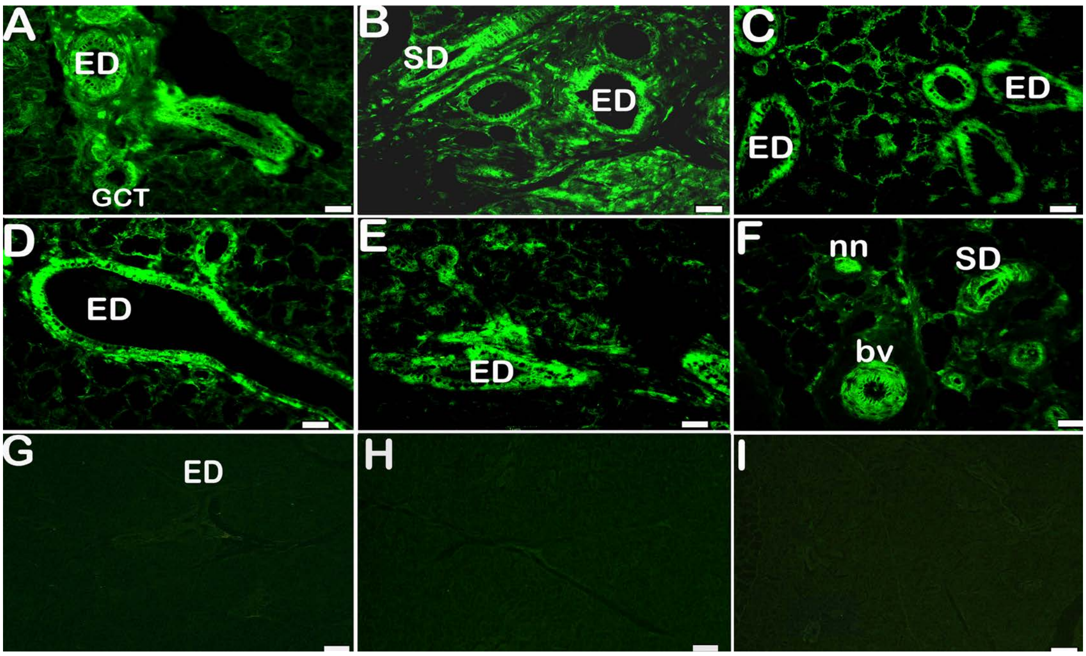

Fig. 4.- Immunofluorescence images showing labelling for CTLA-2 alpha in salivary glands. (A-F): Positive signals are seen in cells of excretory ducts (ED); striated ducts (SD) and granular convoluted tubule (GCT) in submandibular gland and in interlobular artery in smooth muscles of tunica media (bv) and nerve bundles (nn) but is not observed in control sections (G-I). Scale bar A-F: 100 µm; G-I: 200 µm.

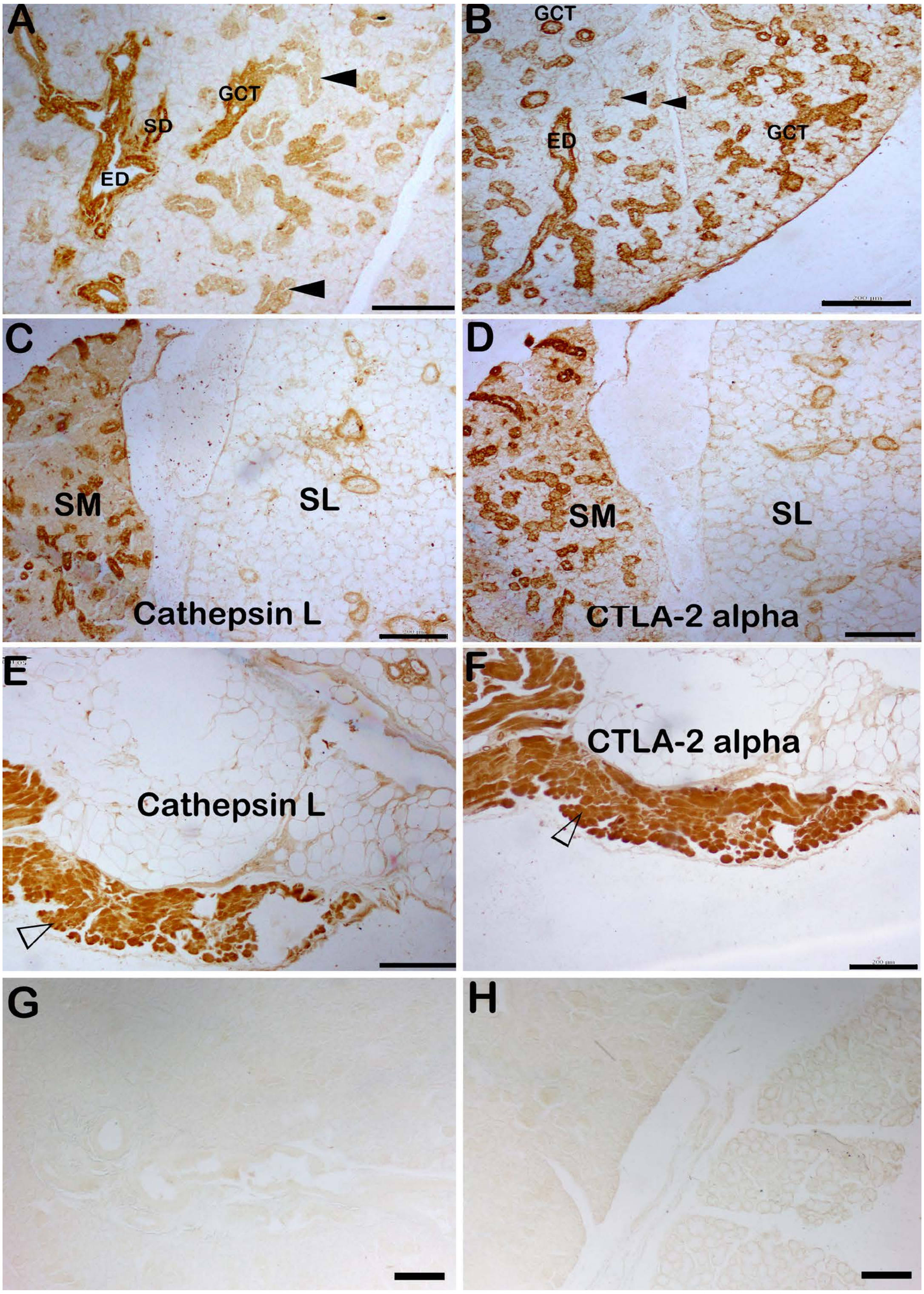

Fig. 5.- Photomicrographs of comparable immunostaining pattern for cathepsin L and CTLA-2 alpha. (A-B): Show faint immunoreactivity for cathepsin L in intercalated (ID) ducts (solid arrow heads) but strong in granular convoluted tubules (GCT), striated ducts (SD) and excretory ducts (ED) of submandibular gland. Similar distribution pattern of immunoreactivity for cathepsin L (C) and CTLA-2 alpha (D) is seen in serial tissue sections of submandibular (SM) and sublingual (SL) salivary glands and skeletal muscles (Open arrowhead) in the interstitial glandular stroma (E-F). Immunoreactivity is not seen in control sections incubated with 10% normal serum in the place of anti-cathepsin L antibody (G) and anti-CTLA-2 alpha antibody (H). Scale bar A-H: 200 µm.

quired to demonstrate colocalization of CTLA-2 alpha and the neurotransmitters such as neuropeptide Y in tissues, particularly dopamine beta hydroxylase, a marker for adrenergic neuropeptide Y nerve fibers of sympathetic origin.

Furthermore, CTLA-2 alpha and cathepsin L were detected in bundles of nerve fibers and skel-

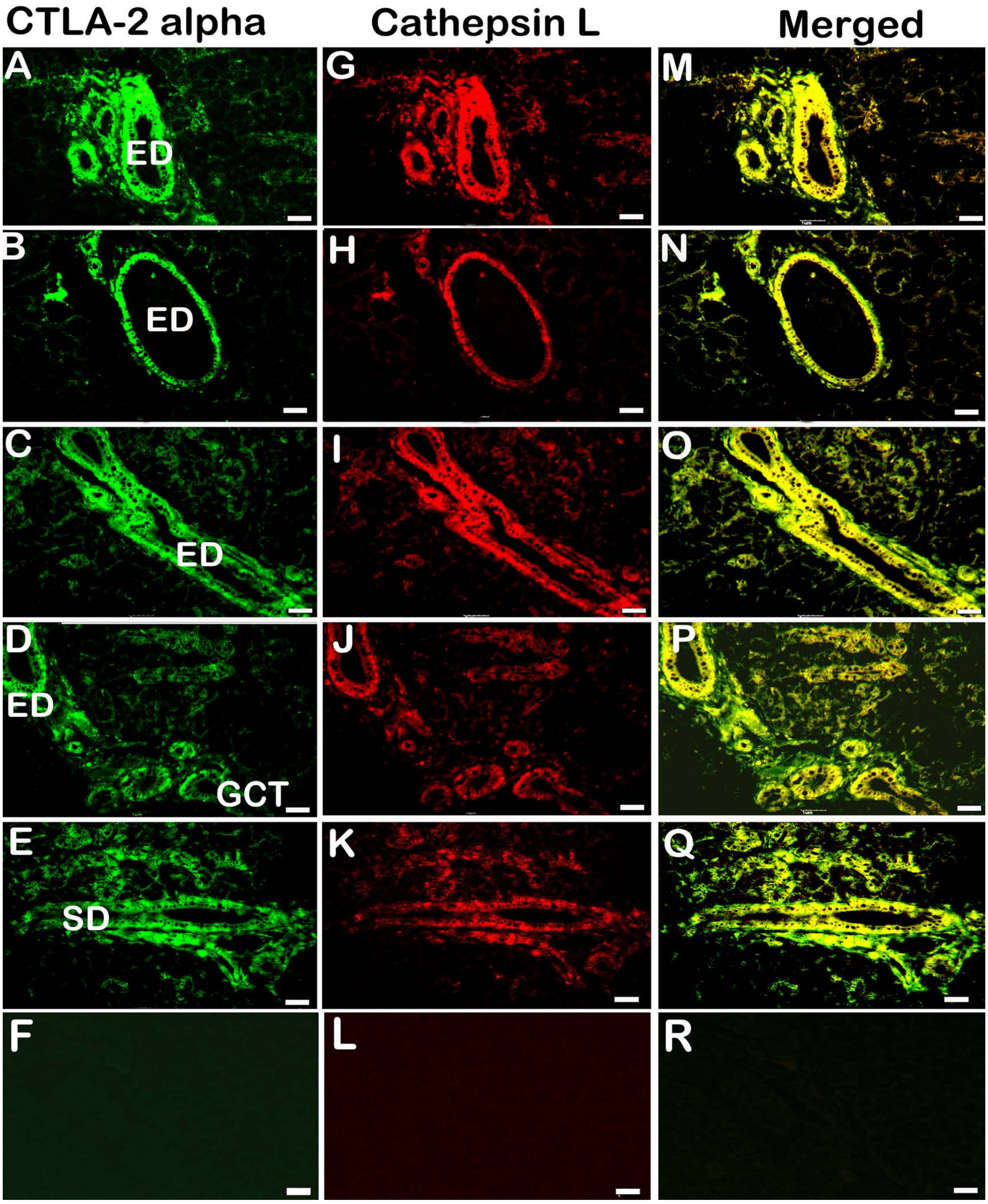

Fig. 6.- Immunofluorescence images demonstrating cathepsin L and CTLA2 alpha double labelling immunoreactivity in ductal epithelial cells of submandibular gland. The first column (A-E) shows labelling for CTLA-2 alpha (green; FITC), the second, (G-K) cathepsin L (red; TRITC) and the third (M-Q) merged (Yellow) images of submandibular gland. Strong immunoreactivity for both CTLA-2 alpha and cathepsin L is seen in cells of excretory ducts (ED), striated ducts (SD) and granular convoluted tubule (GCT). Immunoreactivity is not seen in the control sections (F, L, R) incubated with 10% normal serum in the place of the primary antibodies. Scale bar A-R: 100 µm.

etal muscles used for mastication. CTLA-2 alpha has never been reported before in skeletal muscles, however, cathepsin L has been found to localize in all types of muscle fibers in the rabbit (Taylor et al., 1987), implicating its role in catabolism of myofibrillar component. The observation that muscular action (mastication) of skeletal muscles triggers the reflex to release neuropeptides (Dawidson et al., 1996) is suggestive of the role of CTLA-2 alpha and cathepsin L in bioprocessing of neuropeptides in skeletal muscles. However, their main function in the muscles remains to be established.

In conclusion, this study shows that CTLA-2 alpha is localized in the duct system, blood vessels and nerve bundles in major salivary glands,

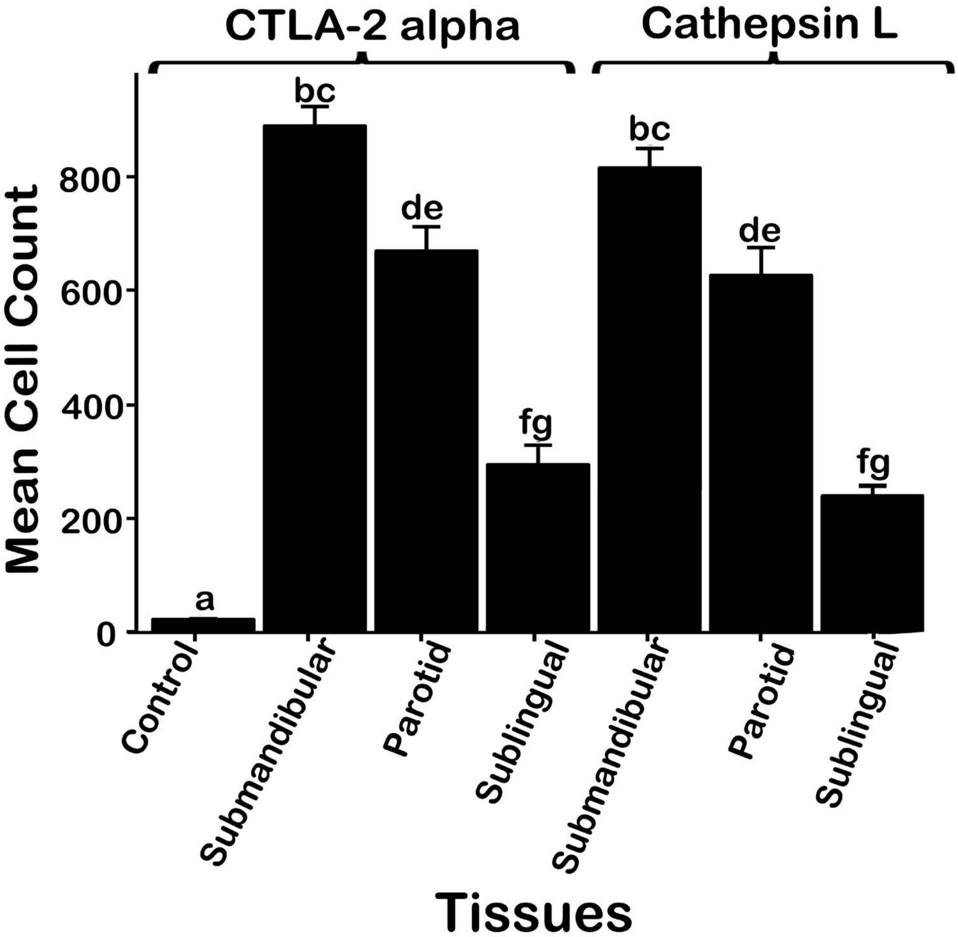

Fig. 7.- Graph showing cell count for CTLA-2 alpha and cathepsin L immunoreactive cells in submandibular, parotid and sublingual salivary glands using Image J. Cell count was performed on sections not incubated with the primary antibodies (Control), and on sections of submandibular, parotid and sublingual salivary glands incubated with the primary antibodies (CTLA-2 alpha and Cathepsin L) at 10,000 µm2 area for each tissue section in ten microscopic fields in five slides. All data were averaged and analysed for statistical significance of means by two-way ANOVA using R statistical software version 4.3.1. Bars with different letters were found to be statistically significantly different at P < 0.05.

concurrently with cathepsin L. The colocalization indicates that CTLA-2 alpha and cathepsin L perform specific functions in physiological processes of salivary glands and opens new avenues for understanding the fine equilibrium between synthesis and secretion of CTLA-2 alpha and cathepsin L potentially for targeting CTLA-2 alpha in drug development for control of oral diseases.

LIMITATION OF THE STUDY

Inability to analyze the intensity of immunoreactivity in adjacent cells or structures where one is showing weak while the other is showing strong immunoreactivity. For instance, in submandibular gland immunoreactivity for the antibodies was weak in epithelial cells lining the intercalated ducts, but was strong in granular convoluted tubules, striated and excretory ducts. Density of positive cells was therefore analyzed based on individual glands (submandibular, parotid and sublingual).

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