MOLECULAR ANALYSIS OF CELL STRUCTURES IN THE PITUITARY GLAND OF BIRDS

(鳥類下垂体細胞の分子生物学的研究)

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THESIS ABSTRACT

Accumulation of colloids has been observed in follicular lumen in the anterior pituitary gland of various animal species. However, very little is known about their origin, composition or biological function. This study was therefore conducted to examine the colloids in relation to different types of cells in the pituitary gland of guinea fowl (*Numida meleagris galeata*). The methods used include Hematoxylin and Eosin (H&E), periodic acid-Schiff (PAS), electron microscopy, immunohistochemistry, Western blot analysis, in situ hybridization and polymerase chain reaction (PCR) analyses.

In this study, numerous and randomly distributed extracellular colloidal accumulations were observed in the guinea fowl. They were positive for PAS reaction and showed variability in shape ranging from round, ovoid and elongated. In 1day old birds, colloids (18.000±1.461) were already accumulated in the pars distalis and gradually increased in number with age ($r = 0.964$; $P<0.0001$). The beginning of colloidal accumulations in the guinea fowl occurred parallel with the development of folliculostellate (FS) cells. Both colloids and FS cells were observed to appear first on or near the posterolateral region in caudal lobe and increased in number towards the periphery. FS cells were frequently found associated with formation of colloids and capillaries. With age FS cells increased and aggregated to each other around a colloid-containing follicle with concurrent formation of gap junctions between their lateral membranes.

Experiments were conducted to assess the biochemical composition of colloids by staining pituitary sections with antiserum to clusterin protein. Positive immunoreactivity was not only evident in the colloids but also in the cytoplasm of FS cells and endocrine cells. Clusterin is shown to contain putative amphipathic α-helices that mediate hydrophobic interactions with numerous types of molecules and may be involved in

clearance of cellular debris caused by cell injury or death. To assess this function *in vivo*, the full-length cDNA encoding guinea fowl clusterin was cloned and its synthesis and expression pattern in specific cell types in pituitary was examined. Quantity of clusterin mRNA expressed in pituitary and endocrine tissues was quantified by real-time PCR. Highest levels were detected in gonads. In situ hybridization showed clusterin mRNA signals in endocrine cells and FS cells.

Clusterin is reported to have a relationship with apoptosis. Apoptosis in the anterior pituitary is a frequent event in which unwanted cells are eliminated without affecting neighbouring cells. The different cell types in anterior pituitary behave as dynamic populations and the gland maintains a continuous renewal of cells to ensure a dynamic balance between cell division, differentiation, growth arrest and apoptosis. The link between apoptosis and the formation of colloids in the guinea fowl pituitary and the relationship of clusterin accumulation in the colloids was investigated. Apoptotic cells detected by ssDNA immunohistochemistry were observed in the whole anterior pituitary preferentially near colloid masses. Simultaneous expression was performed to determine whether clusterin mRNA and apoptosis within anterior pituitary concerns the same cell. It was found that clusterin mRNA was not expressed by apoptotic cells but in neighbouring surviving cells. At electron microscopic level many endocrine cells at different stages of apoptosis were found phagocytosed by FS cells. These findings suggest that clusterin is produced by endocrine cells for cytoprotection before cell death. Apoptoting endocrine cells are phagocytosed by FS cells and digested by their lysosomal enzymes. Clusterin produced by FS cells may interact and aggregate with molecules, proteins and peptides resulting from lysosomal digestion in FS cells and subsequently become stored in the colloid as residual body.

Megalin/LRP-2, a member of the low density lipoprotein (LDL) receptor family has been shown to bind clusterin with high affinity (Kd=14nM) and mediate its endocytosis and lysosomal degradation in cultured cells. In this study, the expression pattern of megalin in specific cell types in the pituitary was investigated. Quantity of megalin mRNA expressed in pituitary and other endocrine tissues was estimated by real-time PCR. High levels were detected in kidney and pituitary. In situ hybridization and immunohistochemistry showed megalin expression in FS cells. At the ultrastructural level, immunogold labeling of megalin was evident in the vicinity of colloids. A Western blot analysis also detected intact megalin from protein extract of the pituitary gland. These results suggest that Megalin may drive ingestion of apoptotic endocrine cells through interaction with clusterin complexes in FS cells, and hence providing a potential mechanism for receptor mediated uptake of degenerating endocrine cells and secretion of colloids.

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LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATION	DESCRIPTION
µl	microliter
µm	micrometer
cDNA	complementary DNA
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EM	electron microscopy
FS cells	folliculostellate cells
H&E	hematoxyline and eosin
HRP	horseradish peroxidase
im	intramuscular
iv	intravenous
Kb	kilobase
kDa	kilodalton
M	molar
mg	milligram
min	minutes
ml	milliliter
mm	milliliter
mM	millimole
Mol	mole
mRNA	messenger RNA
nm	nanometer
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid-Schiff
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	hydrogen ion concentration
pm	picomole

RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription PCR
SDS	sodium dioucyl sulphate
SSC	standard saline citrate
ssDNA	single stranded DNA
TBS	tris-buffered saline
TEMED	N,N,N',N',-tetramethylethylenediamine
UTP	uridine triphosphate
v	volt
X	times

CHAPTER 1

1.0 GENERAL INTRODUCTION

1.1 BACK GROUND INFORMATION

The different cell types in the pituitary gland behave as dynamic populations. The gland maintains a continuous renewal of cells to ensure a dynamic balance between cell division, differentiation, growth arrest, and apoptosis. The anterior pituitary consists of more or less complete cell clusters of hormone-secreting (granular) cells and folliculostellate (FS) cells; agranular cells that surround a colloid-containing follicle. The cells are arranged to allow intercellular communication among the different cell types. Colloid-containing follicles are frequently encountered in the anterior pituitary. They have been reported within the pars distalis and pars intermedia (Benjamin, 1981) and in the pars tuberalis (Kameda, 1990).

They occur in various animal species including cattle (Bassett 1951), bat (Anthony and Gustafson 1984), human (Ciocca et al. 1984), guinea pig (Kameda 1990; 1991), pig (Kubo et al. 1992) and viscacha (Mohamed et al. 2000). Their presence in the vertebrate pituitary has been described as normal component structures of the gland. Their size, shape and frequency of occurrence vary greatly with animal species, age, sex and season of the year (Benjamin 1981; Anthony and Gustafson 1984; Kameda 1991; Mohamed 2000). In addition to their presence within the anterior pituitary, colloids have been described to occur within the residual lumen in many species that retain this space as a patent hypophysial cleft throughout adulthood (Selye 1943; Bassett 1951;

Vanha-Pertulla and Arstila 1970). Despite of their widespread occurrence, little information is available with regard to their origin, composition and function.

Various theories have been proposed regarding the mode of colloid formation. Repeated observation of cell fragments within colloid suggests that colloidal materials arise as a by-product of cellular degradation (Horvath et al., 1974; Ciocca and Gonzales, 1978). An alternative hypothesis suggests that colloid is derived at least in part from interstitial fluid of the pars distalis (Villa-Porcile, 1972; Correr and Motta, 1981). These colloids are found to be completely surrounded by FS cells and therefore it is generally accepted that the major part of colloids is first produced by FS cells and subsequently get released into the follicular lumen (Kagayama, 1965; Fukuda, 1973; Kameda, 1991). Some endocrine cells such as those which produce growth hormone (GH), prolactin (PRL) and luteinizing hormone (LH) are also observed to be closely associated with colloid follicles in the anterior pituitary (Kameda, 1991; Ogawa et al., 1996). Such a phenomenon suggests the existence of a relationship between the colloid-containing follicles, the FS cells and the hormone secreting cells of the pituitary gland. However there is no thorough and systematic analysis to demonstrate the origin of pituitary colloids and the mode of their secretion *in vivo*.

It should be noted however that the FS cells which presumably produce the colloids, contain no secretory granules and are considered to be non-secretory cells of the anterior pituitary (Ogawa et al., 1996). However, the FS cells produce many paracrine secretions that influence adjacent endocrine cells. Such secretions include 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). FS cells are also reported to exhibit phagocytosis by acting as scavenger cells of the anterior pituitary (Inoue et al., 2002). The phagocytotic function of these cells has been clearly demonstrated in the pituitary gland of estrogen-deficient rats, which is known to induce apoptosis of prolactin cells (Drewett et al., 1993). These findings indicate that FS cells are multifunctional cells of the anterior pituitary. Yet the major function of FS cells remains to be established.

The occurrence of many colloidal accumulations in the anterior pituitary of various animals may be indicative of their function. Characterization of the colloids that occur in the anterior pituitary of senescent porcine helped to shed light on the composition of colloids. Ogawa et al., (1997) purified the colloids by centrifugation and percoll gradient. The isolated colloids were solubilized in sodium dodecyl sulfate (SDS) lysis buffer after treatment with exoglycosidase. Analysis using SDS-polyacrylamide gel electrophoresis showed that clusterin and the glycosylated albumin fragments were the major glycoproteins that occur in the colloids. These findings led to the question of the biological significance and the origin of clusterin in the pituitary colloids.

Clusterin is a heterodimeric, disulfide-linked glycoprotein constitutively synthesized and secreted by a wide variety of cell types (Jenne and Tschopp, 1992; French et al., 1993; Rosenberg and Silkensen, 1995; Mahon et al., 1999). Clusterin gene has a single functional promoter. Transcription generates a single full-length mRNA of about 1.6 kb which is translated on membrane-bound ribosome (Rowling and Freeman, 1993) and is targeted to the endoplasmic reticulum by a hydrophobic leader sequence for initial

removal of signal peptide. Proteolytic cleavage into two α- and β-subunits of 40kDa each, dimer assembly and final glycosylation occur in the trans-Golgi network, resulting into an extracellular mature secreted protein, held together by a unique five disulfide bond motif (Wong et al., 1993; Yang et al., 2000; Jones and Jomary, 2002). However, it has been reported the existence of inactive, uncleaved, nonglycosylated clusterin isoform which is expressed in the cytoplasm and nucleus (Leskov et al., 2003; O'Sullivan et al., 2003).

Clusterin is shown to have a relationship with apoptosis. It is associated with cell survival within tissues (French et al., 1994, Koch-Brand and Morgan, 1996) and is induced under low toxic stimuli to exert cytoprotective effects on damaged or stressed cells. However, under high level of cytotoxic stress, uncleaved, nonglycosylated clusterin isoforms accumulate in the nucleus and contribute to cell death (Leskov et al., 2003, Trougakos and Gonos, 2004). Activation of apoptosis is under the control of a network of inter-related signals that originate both from the intracellular and extracellular milieu. Apoptotic cells are morphologically characterized by cell shrinkage, cytoplasmic blebbing, chromatin condensation and formation of membrane-bound fragments of the nucleus called apoptotic bodies (Wyllie et al., 1984). In the anterior pituitary, there is high rate of cell death process by apoptosis. On average, each anterior pituitary cell undergoes either mitosis or apoptosis once every 63 days depending on the endocrine status of the animal (Haggi et al., 1986; Oishi et al., 1993; Nolan et al., 1998; Yin and Arita, 2000). The equilibrium of such processes is maintained by a continuous renewal of cells and therefore the pituitary gland exhibits apparent cyclic pattern of cell turnover.

Megalin/LRP-2, a member of the low density lipoprotein (LDL) receptor family has been shown to bind clusterin with high affinity (Kd=14nM) and mediate its endocytosis and lysosomal degradation in cultured cells (Kounnas et al., 1995). Megalin is located in clathrin coated pits (Kerjaschki et al., 1984; Kerjaschki and Farquhar, 1993) where it serves as an endocytic receptor for a number structurally and functionally diverse proteins, polybasic drugs (Moestrup et al., 1995; Cui et al., 1996) and several polypeptide hormones found in the glomerular filtrate (Orlando et al., 1995). it plays a role in various biological processes including the reabsorption of filtered proteins by cells of the proximal tubule (Moestrup et al., 1994; 1995; Orlando et al., 1995), endocytosis of proteins by yolk sac epithelia (Leung et al., 1989), clearance of proteases and protease inhibitor complexes from the alveolar space by type II pneumocytes (Poller et al., 1995; Stefansson et al., 1995) and in endocytosis and transport of clusterin and its complexes with the amyloid □-peptide across vascular membrane, including blood-brain barrier and blood-cerebral spinal fluid barrier at the choroid plexus. However, its expression and functional role in the pituitary gland has not been demonstrated.

1.2 JUSTIFICATION

Occurrence of colloid in the anterior pituitary has been studies in 43 species of birds and most numerous and largest colloid masses were observed in the guinea fowl [*Numida meleagris*] (Mamba et al., 1989). Therefore, the guinea fowl appears to provide an excellent model for the study of histophysiologic role of pituitary colloids. This observation has led to the investigation of colloids in these birds taking into consideration that little information is available with regard to their origin, composition

and function. In addition, there is no thorough and systematic analysis to demonstrate the origin of pituitary colloids and the mode of their secretion *in vivo*.

FS cells are positive for S-100 protein and produce many cytokines or growth factors such as interleukin-6 (IL-6), leukemia inhibitory factor (LIF), basic fibroblastic growth factor (bFGF) and endothelial growth factor (VEGF). In this context, it is generally accepted that FS cells regulate endocrine cells through these factors. FS cells also exhibit phagocytosis and are known to act as scavenger cells in the anterior pituitary. In addition to these functions, FS cells are considered to have some unknown functions. To reveal the biological functions of FS cell, there is a requirement to perform morphological studies in order to obtain new findings on the role of FS cells.

Previous studies show that clusterin is a major glycoprotein that occurs in the colloid. However, these data lead to a question of the biological importance and the origin of clusterin in pituitary colloids. In conjunction with the identification of clusterin in colloids, concerns are raised regarding the mechanism of clusterin accumulation in pituitary colloids. Clusterin is also shown to have a relationship with apoptosis in that its expression is enhanced in the face of tissue degeneration. However no study that demonstrates the role of clusterin in apoptosis *in vivo*. *In vitro* studies also show that clusterin binds megalin with high affinity and that pituitary colloids contain clusterin. However, the expression pattern of megalin and its functional implications in the pituitary gland has not been demonstrated.

1.3 OBJECTIVES

In view of the above justification, this study had to meet the following objectives:-

(i) To perform morphological studies on anterior pituitary cells and morphormetric studies on colloid structures using PAS, H&E, immunohistochemistry and electron microscopy so as to obtain new findings.

(ii) To examine the pattern of clusterin mRNA expression by quantitative real-time PCR and in situ hybridization so as to determine the specific cell types synthesizing clusterin in the anterior pituitary. Clusterin protein localization was investigated by immunohistochemistry.

(iii) To apply light and electron microscopic techniques so as to examine the phagocytotic activity of FS cells and the relationship of clusterin accumulation in pituitary colloids.

(iv) To examine the pattern of megalin expression in the anterior pituitary so as to determine its functional implication in colloidal accumulation.

CHAPTER 1I

2.0 LITERATURE REVIEW

2.1 EARLY HISTORY OF ENDOCRINE SYSTEM

The first hints of endocrine-like systems did not emerge until the mid-nineteenth century. Our knowledge of the natural history, ecology, ethology, population biology, physiology, and cell and molecular biology of birds is more extensive than for any other class of vertebrates but lags behind that of mammals and fish (Konish et al., 1989). There are significant advantages for experimental investigations of wild species in the field that address adaptations to changes in environmental conditions. Birds are widely distributed, highly diversified, and exhibit behavior and social organizations equal in complexity to mammals, yet they are generally more conspicuous and approachable in natural environments. Moreover, they are diurnal, hardy, and easily withstand more invasive investigations using capture or even surgery (Oring et al., 1988), making field observations tractable (Wingfield and Farner, 1993). Certain species thrive in captivity allowing experimental procedures under rigorously controlled conditions. These attributes make birds excellent subjects in many areas of biological research. And thus, it is not surprising that investigations of birds, including wild species, have made major contributions to biology in general.

2.2 THE AVIAN PITUITARY

2.2.1 Gross structure and subdivisions

Avian neuroendocrine and endocrine systems are very similar to those of mammals and the extensive literature on avian models goes back to the very beginnings of endocrinology. The pituitary gland of birds has two fundamental divisions: the neurohypophysis which is an extension of the brain and the adenohypophysis derived from oral ectoderm.

2.2.2 The neurohypophysis

Neurohypophysis also called the posterior lobe consists of the median eminence, infundibulum and pars nervosa. It is of neural origin and arises from down-growth of the neural ectoderm of the hypothalamus. It is actually the termination of infundibular recess of the third ventricle and consists largely of fibers of the supra-optico–paraventriculo–hypophysial tract, specialized glial cells, pituicytes, and ependymal cells but no neuron cell bodies. The axons descend from two nuclei farther up in the brain, the paraventricular nucleus and the supraoptic nucleus. Their neurons synthesize the peptide hormones oxytocin and antidiuretic hormone (ADH = vasopressin). Each neuron synthesizes only a single type of hormone but both nuclei have neurons for both. The hormones are packaged into secretory granules and transported down the axons to the pars nervosa. Here they accumulate in the expanded end of the axons which lie up against capillaries. When the neuron fires, its hormone is released as though it were a neurotransmitter and is and picked up by the capillary. The ends of the axons are visible in suitable preserved preparations as packets of secretory granules and are called Herring bodies. This name also applies to clusters of granules that pile up along the axon as they migrate down in the infundibulum as well as the posterior lobe.

Wingstrand (1951) work on avian species was among the first to report that fibers from the supraoptic nucleus can be traced to the neurohypophysis.

2.2.3 The adenohypophysis

This is also known as the anterior pituitary. It has a relatively large pars distalis (caudal and cephalic lobes) and a smaller pars tuberalis. Birds are unique among other non-mammalian vertebrates in that their pituitary lacks a pars intermedia (Mikami, 1986). The anterior pituitary is composed of cords of glandular epithelial cells separated by the numerous sinusoidal capillaries of the secondary capillary plexus and is not directly innervated by hypothalamic nerves, but only by autonomic fibers from the carotid plexus.

Development and differentiation of the adenohypophysis is divided into three major stages: the gland commitment from the upward evagination of the ectodermal lining the primitive oral cavity, formation of the Rathke's pouch and emergence of the terminally differentiated cell types. Sasaki et al., (2003) demonstrated that specific areas in the epithelium of Rathke's pouch differentiate into specific regions of the adenohypophysis at 20 days. The walls of Rathke's pouch are divided into upper and lower parts of the cranial and caudal wall. The cephalic lobe is mainly assembled by the proliferation of parenchymal cells in the areas $A_2 + A_3 + P_2$ of Rathke's pouch epithelia at 3 days of incubation. The caudal lobe is derived from $A_1 + P_1 + P_3$. The pars tuberalis is derived from $A_1 + A_2$. Thus, the avian adenohypophysis is established at 13 days and the blood supply to the pars distalis is established at 20 days. At day 3 , the cephalic lobe and caudal lobe of the pars distalis and the pars tuberalis of the avian adenohypophysis are derived from specific areas of the cell cords of Rathke's pouch.

2.2.4 Vascular circulation

Blood supply to the pituitary gland derives from two sets of arteries: inferior and superior hypophyseal arteries. Superior arteries give off a capillary plexus in the pars tuberalis. Portal vessels drain this plexus and supply the capillaries in the pars distalis. In the pituitary stalk, the capillary loops arising from and coming back on the capillary plexus are surrounded by hypothalamic nerve endings. These nerve endings release the hypothalamic hypophysiotropic factors into the bloodstream. The adenohypophysial portal circulation supports the neural vascular link between the hypothalamus and the pituitary.

2.2.5 Nerve supply

The hypothalamo-hypophysial tract consists primarily of nerve fibers from the supraoptic and paraventricular nuclei carrying vasopressin, oxytocin and their neurophysins to the posterior lobe of the pituitary where the homones are released into the capillaries. The tubero-infundibular tract originating from the neuroscretory neurons which produce hypophysiotropic hormones projects from several nuclei to the median eminence where the hormones are released into the hypophyseal portal vascular system. The adenohypophysis has no direct nerve supply, apart from the small sympathetic nerve fibers which are associated with and presumably innervate capillaries (Kovacs and Horvath, 1986). In some species the intermediate lobe has rich innervation (Cox et al., 1978). Although neural connections may affect blood flow to the adenohypophysis, it is unlikely that they are involved in the regulation of adenohypophysial hormone secretion which is predominantly neurohumoral.

2.2.6 Functional anatomy of the adenohypophis cells

The adenohypophysis consists of at least five major cell types that are phenotypically characterized by the trophic hormones they synthesize and secrete. These cell types include (A) **chromophobes** which stain poorly and appear clear or white in tissue sections. Together, the three subpopulations of chromophobes make up about 50% of the epithelial cells in the pars distalis. They include (1) the undifferentiated nonsecretory cells, which may be stem cells; (2) the partly degranulated chromophils, which contain sparse granules; and (3) the folliculostellate cells, the predominant chromophobe type, which form a stromal network that supports the chromophils. The folliculostellate cells may have some phagocytic functions. (B) **Chromophils**; these hormone-secreting cells of the pars distalis stain intensely owing to their abundant cytoplasmic secretory granules in which their hormones are stored.

There is a specific cell type for each hormone. Usually larger than chromophobes; the chromophils are subdivided into two classes: (i) Acidophils: These cells secrete simple proteins. They stain intensely with eosin and orange G, but not with Periodic-Acid Schiff (PAS). They are more abundant in the periphery of the gland and are usually smaller than basophils and their granules are larger and more numerous. The acidophils include two major types of hormone-secreting cells: somatotropes, which produce growth hormone (GH, somatotropin), and mammotropes, which produce prolactin. (ii) Basophils: These cells, which stain with hematoxylin and other basic dyes, secrete glycoproteins and are PAS-positive. They are more abundant in the core of the gland and are usually larger than acidophils, with fewer and smaller granules.

The three major types of hormone-producing basophils produce four major hormones. (1) Each of the two types of gonadotropes produces a different gonadotropin. The first produces follicle-stimulating hormone (FSH) and the second produces luteinizing hormone (LH; called interstitial cell-stimulating hormone [ICSH] in males). (2) Corticotropes produce adrenocorticotropin (ACTH). (3) Thyrotropes produce thyrotropin (thyroid-stimulating hormone, TSH). The pars tuberalis contains mostly gonadotropes and many capillaries of the primary capillary plexus of the hypophyseal portal system.

These cell types do not arise simultaneously but rather differentiate during embryonic development in a temporally distinct manner that is similar across vertebrate species (Kioussi et al., 1999; Savage et al., 2003). The first pituitary marker to appear in rodents and chickens is the alpha-glycoprotein, subunit alpha-GSU (Kameda et al., 2000) that is common to luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH). However, corticotrophs, which produce adrenocorticotropic hormone (ACTH), are the initial hormone-secreting cell type to differentiate in chickens, rats, and mice (Sasaki et al., 2003). The next cell types to appear are gonadotrophs, which secrete LH and FSH, and thyrotrophs, which produce TSH (Maseki et al., 2004; Nakamura et al., 2004). The final two cell types to differentiate in the developing pituitary gland are somatotrophs, which secrete growth hormone (GH), and lactotrophs, which produce prolactin (PRL) (Woods and Porter, 1998; Sasaki et al., 2003).

2.2.7 General structure of folliculostellate cells

In the adenohypophysis of higher vertebrates, the presence of folliculostellate cells (FS cells) has been described in addition to the hormone-producing cells. These cells were first identified by electron microscopy. They are non-hormone producing cells and are characterized by their star shaped appearance and follicle formation (Rinehart and Farquhar, 1953; Kagayama, 1965). FS cells were difficult to observe by light microscopy until the finding that immunocytochemistry could determine the presence of S-100 protein, which is contained specifically in FS cells in the mammalian pituitary; hence known as a marker protein of FS cells (Nakajima et al., 1980). FS cells have been suggested as a major type of agranular cells that may be responsible for local regulation of the functions of the anterior pituitary of mammals (Rinehart and Farquhar, 1953; Shirasawa et al., 1983; Ogawa et al., 1996; Inoue et al., 1999). The presence of S-100 immunoreactive FS cells has also been demonstrated in the pituitary gland of birds (Van Nassauw et al., 1987, Luziga et al., 2006).

In normal rat, FS cells are in contact with many kinds of endocrine cells; however, castration leads to a characteristic change of morphology in gonadotrophs. FS cells are observed to specifically surround hypertrophied gonadotrophs (Shirasawa et al., 1983). FS cells also increase in size and number following castration (Inoue et al., 2002). In addition, blood vessels appear well developed and FS cells occasionally make contact with the blood vessels. The contact of the cytoplasmic processes of FS cells with blood vessels is considered to facilitate transport of nutrients or oxygen from blood vessels to the endocrine cells.

2.2.8 The role of folliculostellate cells

Several functions have been ascribed to FS cells including phagocytosis (Stockreef et al., 1986), stem cell function (Yoshimura et al., 1977) and paracrine regulation of hormone secreting cells (Baird et al., 1985). They also produce many substances that influence adjacent 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). In addition to the above functions, FS cells are also involved in the secretion of colloids (Kagayama, 1965; Fukuda, 1973).

Electron microscopic observation have confirmed the light microscopic findings that these colloids are completely surrounded by long cytoplasmic processes originating from FS cells (Luziga et al., 2006), suggesting that the colloids are first produced by FS cells and subsequently released into follicular lumen. Some glandular cells such as those which produce growth hormone, prolactin and lutenizing hormone are also closely associated with colloid follicles in the anterior pituitary of senescent guinea pig (Kameda, 1991) and porcine (Ogawa et al., 1996). In the pars distalis of viscacha two different types of colloid-containing follicles have been demonstrated by electron microscopy, namely: those surrounded by nongranulated follicular cells corresponding to a characteristic follicle and those surrounded by granulated cells (Mohamed et al., 2000). An additional type of colloid-containing follicle surrounded by specific cells that are packed with vesicular inclusions was identified in the pars tuberalis of guinea pigs (Kameda, 1990). Besides the pars distalis and tuberalis, colloids also occur in the pars intermedia (Anthon and Gustafson, 1984; Kubo et al., 1992).

Occurrence of colloid in the anterior pituitary gland has been studied in 43 species of birds. The most numerous and largest colloid accumulations were observed in the guinea fowl; *Numida meleagris galeata* (Mamba et al., 1989). In mammals, colloids have been observed in cattle (Bassett, 1951), bat (Anthony and Gustafson, 1984), human (Ciocca et al., 1984), guinea pig (Kameda, 1990; 1991), pig (Kubo et al., 1992) and viscacha; *Logostomus maximus maximus* (Mohamed et al., 2000). Colloidal accumulations vary greatly in size, shape and frequency of occurrence with animal species, age, sex and season of the year (Benjamin, 1981; Anthony and Gustafson, 1984; Kameda, 1991; Mohamed et al., 2000).

In addition to their presence within the anterior pituitary, colloidal accumulations have also been described to occur within the residual lumen in many animal species that retain this space as a patent hypophysial cleft throughout adulthood (Selye, 1943; Bassett, 1951; Vanha-Pertulla and Arstila, 1970). Various theories have been proposed regarding the mode of colloid formation. Repeated observation of cell fragments within colloid indicates that colloidal materials arise as a by-product of cellular degradation (Horvath et al., 1974; Ciocca and Gonzales, 1978). An alternative hypothesis suggests that colloids are derived at least in part from interstitial fluid of the pars distalis (Villa-Porcile, 1972; Correr and Motta, 1981).

2.2.9 Occurrence of clusterin protein in pituitary colloids

The work of Ogawa et al., (1997) to characterize the colloids from senescent porcine pituitary gland helped to shed light on the composition of the colloids. They purified the colloids by centrifugation and percoll gradient. The isolated colloids were solubilized in sodium dodecyl sulfate (SDS) lysis buffer following treatment with exoglycosidase.

Analysis using SDS-polyacrylamide gel electrophoresis showed that clusterin is the major glycoprotein that occurs in the colloids. Apart from clusterin, albumin fragments were also detected. These findings led to the question of the biological significance and the origin of clusterin in the pituitary colloid.

Recent findings have revealed the mechanism by which clusterin accumulates in pituitary colloids. Luziga et al., (2005; 2006) showed that FS cells express clusterin mRNA and protein. It is also shown that clusterin is produced by glandular cells adjacent to FS cells. Clusterin expression is related to apoptosis. Some endocrine cells such as GH-, PRL-, or LH-secreting cells that were to undergo apoptosis because of aging; they must be phagocytosed by neighbouring FS cells. The clusterin in the phagocytosed cells and that produced by FS cells may interact and aggregate with molecules, proteins and peptides resulting from lysosomal digestion in FS cells and subsequently become stored in the colloid as residual body. This possibility is supported by the fact that many degenerating endocrine cells accumulate around the FS cells which contain colloids (Kameda 1991; Ogawa et al., 1996; Luziga et al., 2006).

2.2.10 Localization of other peptides in the adenohypophysis

A number of substances not initially thought to be of pituitary origin are detectable in the adenohypophysial cells. These peptides have been observed to affect gonadotropin activity and often appeared to have their main derivation in the hypothalamus. The peptides include endothelin (Kanyicska et al., 1991), galanin (Lopez et al., 1991), neuropeptide Y (NPY) in rat (McDonald et al., 1987) and lamb (Sahu et al., 1998), oxytocin, pituitary polyadenylate cyclase-activating polypeptide (PACAP) in rat (Koves et al., 1990; 1991; Perrin et al., 1993) and sheep (Mikkelsen et al., 1995), and substance

P (Brown et al., 1990; Parnet et al., 1990) and also C-type natriuretic peptide (CNP), epidermal growth factor (EGF), interleukin (IL)-6, nerve growth factor (NGF) (Spangelo et al., 1989; Yamaguchi et al., 1990), gastrin-releasing peptide and opioids, and, less certainly, neurotensin (Watanobe and Takebe, 1993), vasoactive intestinal polypeptide (VIP) and opiods (Dragatis et al., 1995). The ability of these compounds to influence LH release in the absence and/or presence of GnRH suggests that the physiological regulation of gonadotropins is a result of interaction of many peptidic factors. The manner in which the peptides interact with GnRH and among themselves, the molecular mechanisms that possibly underlie the processes, and how so many factors are integrated into a physiologically functional system pose fascinating questions.

2.2.11 Pituitary receptors

For peptides to act at the pituitary requires the presence of receptors. In fact, it is often assumed that the presence of receptors implies that the corresponding peptide exerts a physiological influence on the cell. In the pituitary, high-affinity receptors have been found for many of these peptides, although whether receptors are on gonadotropes has not always been determined (Stojilkovic et al., 1992). However, the receptors have sometimes been detected, directly or by measurement of a receptor-mediated activity, on αT3–1 cells, a gonadotrope-derived cell line, providing strong inferential evidence that mature gonadotropes would be similarly endowed. If the receptor to an LH-modulating peptide is not on gonadotropes, but present in the anterior pituitary gland, then a paracrine activity is suggested (Chadio and Antoni, 1989; Gottschall et al., 1990; Takabashi et al., 1991; McArdle et al., 1992; Quinones-Jenab et al., 1997).

2.2.12 Adenohypophisial pathology

Variations in the number, size and distribution of the different cell types in the anterior pituitary are seen in a number of conditions. During pregnancy and lactation the gland may increase in size by up to one third due to a true hyperplasia of prolactin secreting cells. Changes in other cell types occur in some endocrinopathies, following surgical removal of pituitary hormone target organs, and in conjunction with certain drug therapies. The gland also shows age related changes (Kovacs et al., 1981; Pernicone et al., 1992).

The most significant lesions seen in the pituitary gland are benign adenomas (DeLellis, 1989; McKeever and Spicer, 1987). Current classifications identify ten types of pituitary adenoma based on immunocytochemical demonstration of specific hormone production and tumour cell ultrastructure (Kovacs et al., 1981; Taylor, 1986). Tumour cells may be non-secretory or produce one or more hormones: chromophobic adenomas (the most common type) are able to produce any of the anterior pituitary hormones; acidophilic adenomas produce PRL and/or GH; and basophilic adenomas produce ACTH, b -LPH and/or endorphins (Kovacs et al., 1981). TSH, LH or FSH secreting adenomas are uncommon (DeLellis, 1989).

CHAPTER III

3.0 FORMATION AND DEVELOPMENT OF COLLOID CONTAINING FOLLICLES IN THE ANTERIOR PITUITARY

3.1 INTRODUCTION

Extracellular accumulations of colloids have been reported within the pars distalis and pars intermedia in mammals, birds, reptiles, amphibians and fishes (Benjamin, 1981) and pars tuberalis (Kameda, 1990). Their size, shape and frequency of occurrence vary greatly with animal species, sex, age and season of the year (Benjamin, 1981). However little is known about the origin of pituitary colloid despite of their widespread occurrence. Surrounding a colloid-containing follicle, FS cells have been frequently observed and thus considered to be involved in colloidal formation. However little information is available with regard to the precise stimuli promoting their formation and other type of cells forming follicles besides FS cells. Numerous follicles of large size and various shapes with abundant accumulations of colloids occur under natural condition in the anterior pituitary of helmet guinea fowl. The guinea fowl appears to provide an excellent model for the study of biological function of pituitary colloids. This study was therefore designed to investigate colloids in the guinea fowl with the purpose of studying their accumulations and possible connection with FS cells using light and electron microscopy.

3.2 MATERIALS AND METHODS

3.2.1 Source of specimens and tissue preparation

Paraffin embedded pituitary specimens were prepared from 32 helmet guinea fowls aged 1 days to 12 months reared from May, 2003 to July, 2004 at Yamaguchi University, Japan; 36:00 N; 138:00 E, with 3.6-26.5°C, minimum and maximum temperature in 2003 / 2004. To prepare a pituitary for the study, a bird was anaesthetized with Nembutal [pentobarbital] (0.8ml/kg iv) and the pituitary was quickly excised and processed for light or electron microscopy.

3.2.2 Histochemical and morphometric studies

The brain was rapidly exposed and the pituitary gland was excised, sagittally sectioned and processed for light microscopy, fixed in Bouin's fluid, embedded in paraffin, serially sectioned in the horizontal plane at 5μm and stained with H&E and PAS. Structural stereological studies were performed using the optical slides of pituitaries of guinea fowls at different ages. Cross sections stained by PAS were examined with a Zeiss microscope. For morphometric studies the tissue sections were examined using a 40x objective and 10x eyepiece. Ten horizontal sections (5μm thickness) regularly spaced throughout the pituitary of each guinea fowl pituitary were chosen for the numeric analysis of colloid content. For each section all colloid accumulations were counted including both median and lateral areas of pars distalis. The total number of colloid structures was determined for each pituitary. The diameters of the colloidal accumulations counted were measured using an ocular micrometer. Statistical analyses were performed using a two-tailed Student's *t*-test (differences between two groups) or one-way analysis of variance followed by Fisher's PLSD test (Difference between all means). $P<0.05$ was considered significant. Correlation between the number of colloids

and age was investigated by linear regression analysis and the results were expressed as the mean±SE.

3.2.3 Immunohistochemistry

Sections were deparaffinized in xylene then rehydrated through a descending ethanol series to phosphate-buffered saline (PBS). They were immersed in a solution of 0.3% v/v hydrogen peroxide in water for 20 min at room temperature (RT) in order to inhibit endogenous peroxidase activity, and then washed (3×5 min) in 0.01 M PBS, pH 7.4. Sections were incubated with 10% normal goat serum in PBS for 30 min at room temperature (RT) to block non-specific binding. To detect S-100 protein the sections were incubated with polyclonal rabbit anti-cow S-100 protein (DAKO, Denmark, Code No Z0311) diluted 1:400 in PBS and applied to sections for 24 h in a dark, humid chamber at 4 °C. Sections were washed (3×10 min) in PBS, followed by incubation for 30 min at RT with biotinylated goat anti-rabbit IgG (MP Biomedicals, Inc., Germany). The sections were then incubated with streptavidin-peroxidase conjugate for 20 min at RT followed by washing (3×5 min) in PBS. Visualization of binding sites was accomplished by incubating the sections for 3–5 min with a medium containing 0.05%3,3'-diaminobenzidine tetra-hydrochloride, 0.01% hydrogen peroxide and 0.05 M Tris–HCl, pH 7.6. As negative controls, sections were treated as above, except that 1% bovine serum albumin in PBS was applied in place of the primary antisera. The sections were counterstained with Mayer's hematoxylin (Dako) for 30 s, rinsed for 15 min in running tap water and then dehydrated through a graded alcohol series, cleared and mounted in entellan (MERCK, Darmstadt, Germany). Labeling was assessed using a Zeiss microscope (Axiophot 2, Germany).

3.2.4 Electron microscopy

Pituitaries were rapidly excised and sliced into 1mm slabs in a few drops of pre-fixative solution (2% Paraformaldehyde, 2.5%Glutaraldehyde in 0.1M Sodium Cacodylate buffer, pH7.4 with 0.2mM $CaCl_2$). The tissues were then pre-fixed in the same pre-fixative solution for 90 minutes at 4°C with gentle shaking. After a rinse in 0.1M Cacodylate buffer, pH7.4, the tissues were post-fixed in 2%Osmium tetroxide for 90 min at 4°C followed by dehydration in graded alcohol series exchanged with acetone and embedding in epoxy resin (Quetol 653: NISSIN EM, Tokyo). Sections of 1μm thick were cut with diamond knife on ultramicrotome (2088 Ultrotome®V, LKB Bromma) then stained with 1% toluidine blue and evaluated by light microscopy. Areas with follicular lumen were selected for fine trimming and thin sectioning. Ultra thin sections of approximately 70nm thick were cut, mounted on grids and stained with 2% uranyl acetate for 20 min and Lead citrate for 7 min. Specimens were finally examined at 80kV in transmission electron microscope (Hitachi H-7600 TEM Version 3.01).

3.3 RESULTS

3.3.1 Periodic acid Schiff staining and morphometric studies

The gross structure of helmet guinea fowl adenohypophysis is similar to that described previously in other avian species (Mikami 1958). It consists of the pars distalis (caudal and cephalic lobes) and the pars tuberalis which bridges between the median eminence and the pars distalis. The latter two are not connected directly but by the pituitary portal vessel, there being no pars intermedia (Fig. 1).

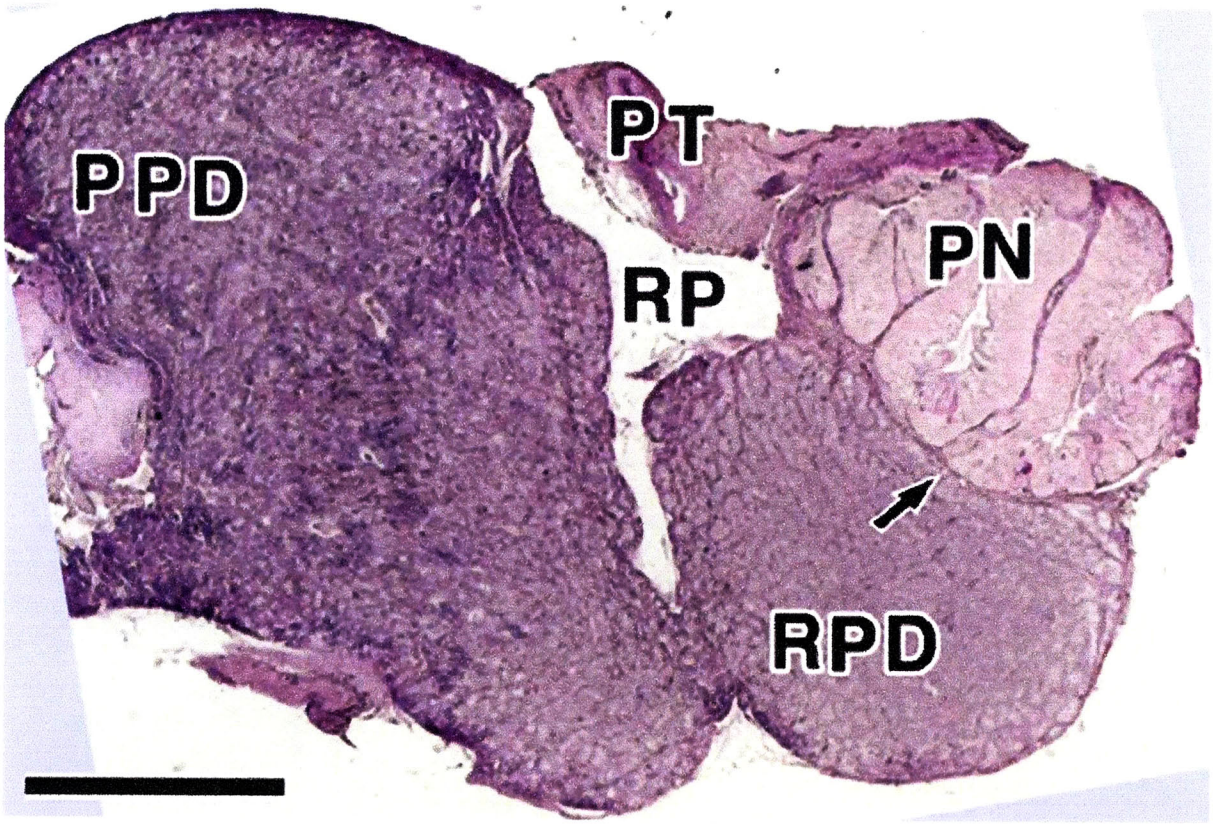

Fig. 1. PAS stained sagittal section of pituitary gland from 1-day-old helmeted guinea fowl illustrating the gross structure. **PPD**: proximal pars distalis; **RPD**: rostral pars distalis; **PN**: pars nervosa; **arrow**: posterolateral region. **PT**: pars tuberalis; **RP**: Rathke's pouch. Scale bar: 0.5mm

PAS reaction was used to demonstrate the occurrence of colloids in the anterior pituitary gland. A few colloids (18.000±1.461) were observed in 1 day old birds (Table 1).

Table 1: The mean number of colloidal accumulations in the pars distalis from helmeted guinea fowls at various ages

Age	Body weight in grams	No:- of birds examined	Average number of colloids in 10 slides (mean±SE)
1 day	30	5	69.5±2.997
1 month	350	5	179.833±3.458
2 months	750	5	264.667±2.985
3 months	1200	5	523.8±5.826
6 months	1550	5	868.333±17.942
9 months	1950	5	1520.667±13.571
12 months	2100	5	2708±38.478
15 months	2200	5	2931.333±29.847

Age difference of mean diameter of colloid deposits was determined for 1 day to 15 months old birds by one way analysis of variance followed by Turkey's test ($P<0.0001$) Means with the same superscripts along the same rows and column are not significantly different at $P> 0.05$.

Table 2: The mean diameter/length of colloidal accumulations (in μm±SE) in the pars distalis of helmeted guinea fowls at various ages

Age	Body weight in grams	Number of birds	Mean diameter of colloid		
			Round	Ovoid	Elongated
1 day	30	5	0.169±0.014	-	-
1 month	350	5	0.263±0.022	-	-
2 months	750	5	0.35±0.025	-	-
3 months	1200	5	0.406±0.024	0.469±0.034	-
6 months	1550	5	0.688±0.036	1.188±0.068	1.575±0.041
9 months	1950	5	1.425±0.041	1.638±0.042	2.188±0.075
12 months	2100	5	1.750±0.155	1.975±0.145	2.638±0.151
15 months	2200	5	2.263±0.078	2.888±0.62	4.188±0.315

In young birds most of colloids were localized in the caudal lobe near the posterolateral region, peripheral zones and very few in the cephalic lobe (Fig. 2A). They increased in number in the areas in 1 month (Fig. 2B) and continued to increase with age ($r = 0.964$; $P<0.0001$). In older birds (above 6months old) the distribution of colloids was random throughout the anterior pituitary (Fig. 2C, D). They showed strong PAS positive, observed at the center of a cells cluster. The cells clusters were bound by a basement membrane in association with capillaries. The average area of the pars distalis per sample section was 1.92±0.087mm^2 in young (below 6 months) and 2.57±0.145mm^2 in older birds (above 6 months). Average diameters of round, oval and length of elongated colloidal deposits were measured and results are shown in Table 2. It was further more observed that, the number of colloidal accumulations increased with body weight ($P<0.001$).

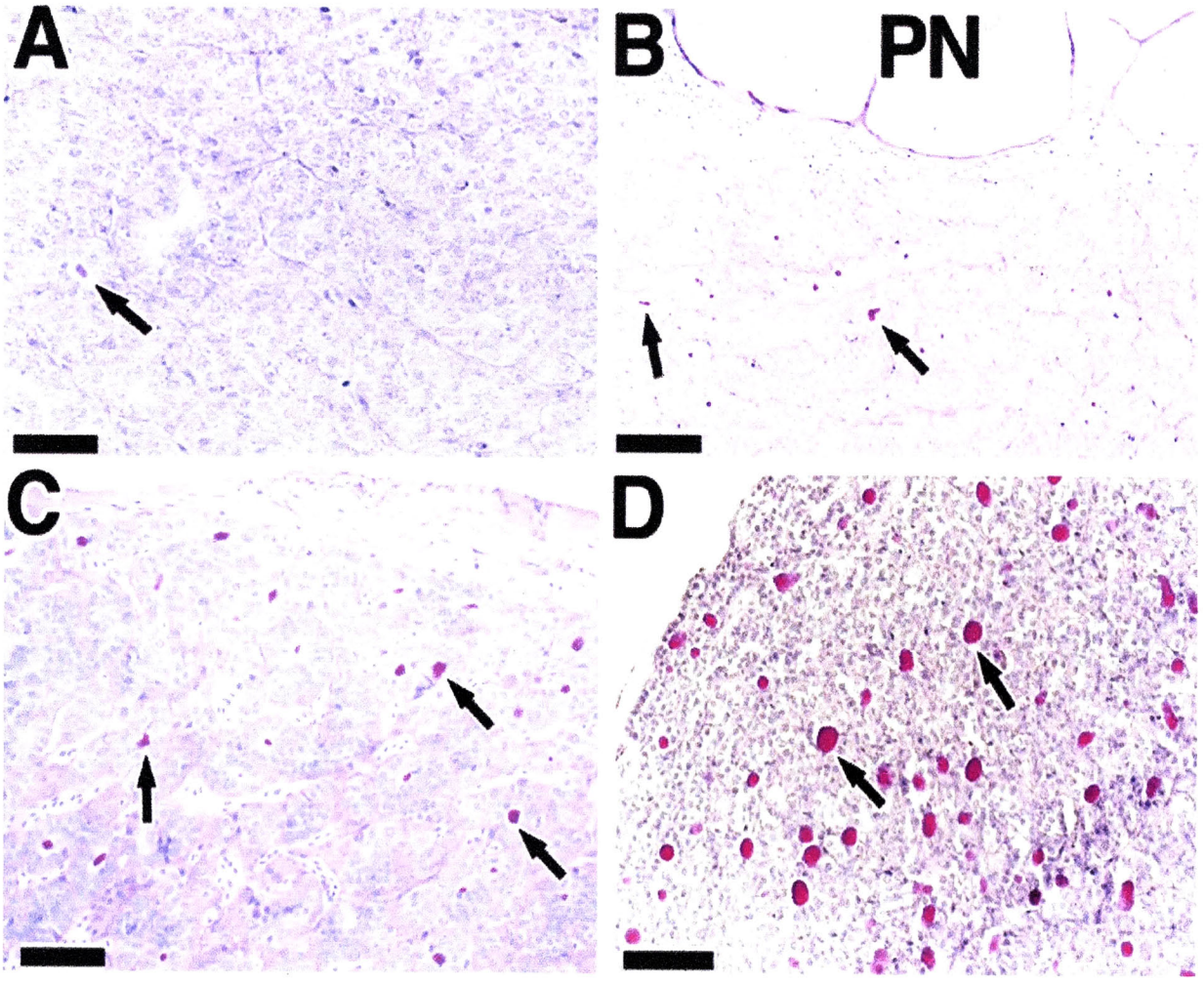

Fig. 2. PAS stained cross sections of pituitary gland of guinea fowl. Colloidal accumulations (arrows) appeared to increase in number with age from (A) 1-day-old bird showing a few colloids, (B) 1-month old, (C) 6-months old and (D) 15-months old birds in which numerous colloids are observed. Scale bar: A, B, C: 70µm; D: 50µm

3.3.2 Immunohistochemical observations

Immunohistochemistry of S-100 protein was performed on the pars distalis of helmet guinea fowl in order to localize FS cells. First anti S-100 immunoreactive cells in young birds was the marginal cell layer. The other population of positive cells was the FS cells found on or near the posterolateral region in the caudal lobe (Fig. 3A, B). FS cells increased in number with age from both ends of the region towards the periphery and then appeared at the center. In older birds, S-100 immunoreactive FS cells appeared in the whole anterior pituitary. The cells extended long cytoplasmic processes into the interstitial spaces of adjacent cells and increased with increasing age, becoming in contact with each other, forming a meshwork of cytoplasmic processes (Fig. 3C, D).

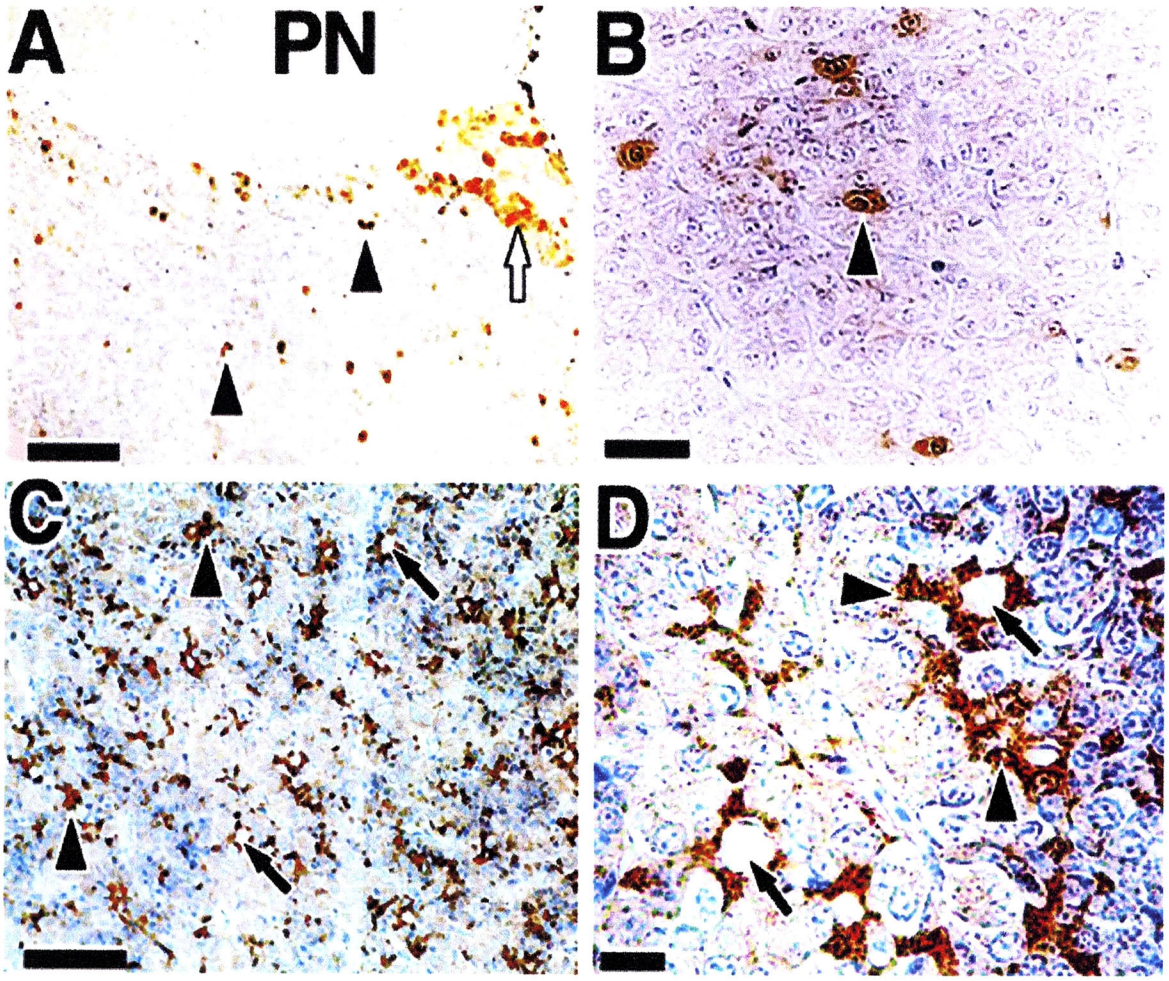

Fig. 3. Anti S-100 protein positive FS cells. Immunostained sections from (A) 1-month-old bird showing positive S-100 protein immunoreactivity in marginal cell layer (open arrow) and FS cells (arrowheads) near or on the posterolateral region and increase towards peripheral zones. Larger magnification of (A) is shown in (B). (C) 15-months-old bird showing numerous FS cell surrounding colloids and extend several cytoplasmic processes to surround adjacent endocrine cells. Scale bar: A, C: 100μm; B, D: 50μm

3.3.3 Electron microscopic observations

Formation of colloid-containing follicles appeared to start within the cytoplasm of a single FS cell. The cell first develops a predominant junction complex lateral to the site of colloid accumulation (Fig. 4A, B) and from one side, the cell extends cytoplasmic processes to surround adjacent granulated cells and extend further to the site of capillary (Fig. 4C, D). FS cells increase in number around colloid by mitosis, at the same time developing junction complexes to join their lateral membranes. Generally, electron microscopic observations agreed well with the light microscopic results. The pars distalis appeared to be composed of distinctly separated lobules resting on a basement membrane and surrounded by capillaries. At the center of a lobule, a well-developed follicle containing electron lucent colloid, almost completely surrounded by a cluster of FS cells was observed. Colloid-containing follicles of various shape and size were encountered. Some appeared round with electron lucent or electron dense colloid and others were elongated or irregular in shape (Fig. 5, 6). Typical FS cells were recognized by electron microscope by their lack of cytoplasmic granules.

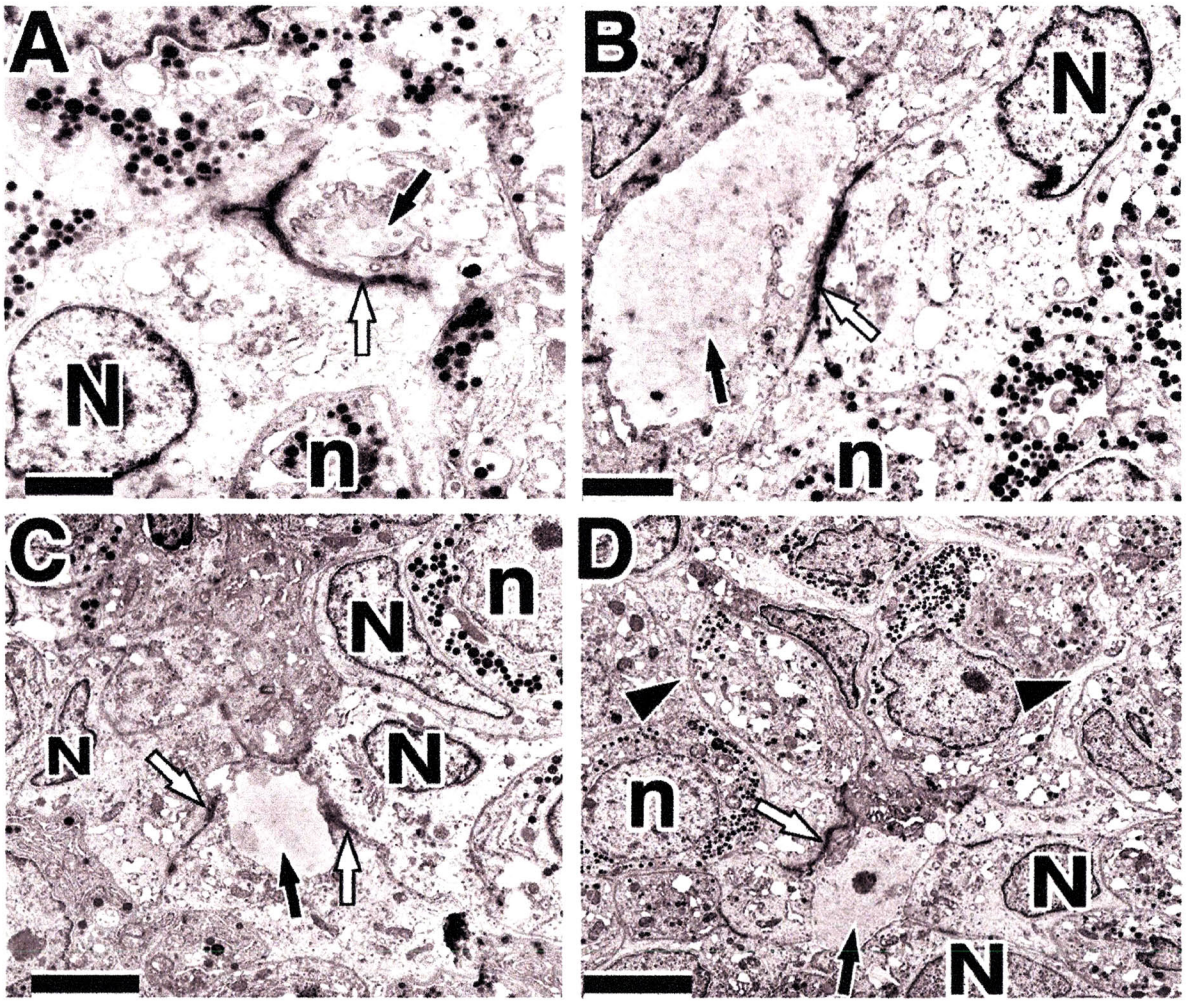

Fig. 4. Electron micrographs of forming colloid-containing follicle. (A) Shows a follicle with scanty colloid (arrow) at initial stage of follicular development in the cytoplasm of FS cell (N). Lateral to that is a prominent junction complex (open arrow). The number of FS cells increase as the follicle develops (B, C). Junctional complexes (open arrows) are also observed along lateral membranes of FS cells. The cells project long cytoplasmic process (arrowhead) through the interstitial spaces of granulated cells (n) to the site of capillaries (**C**). Scale bar: A, B: 500nm; D: 2μm.

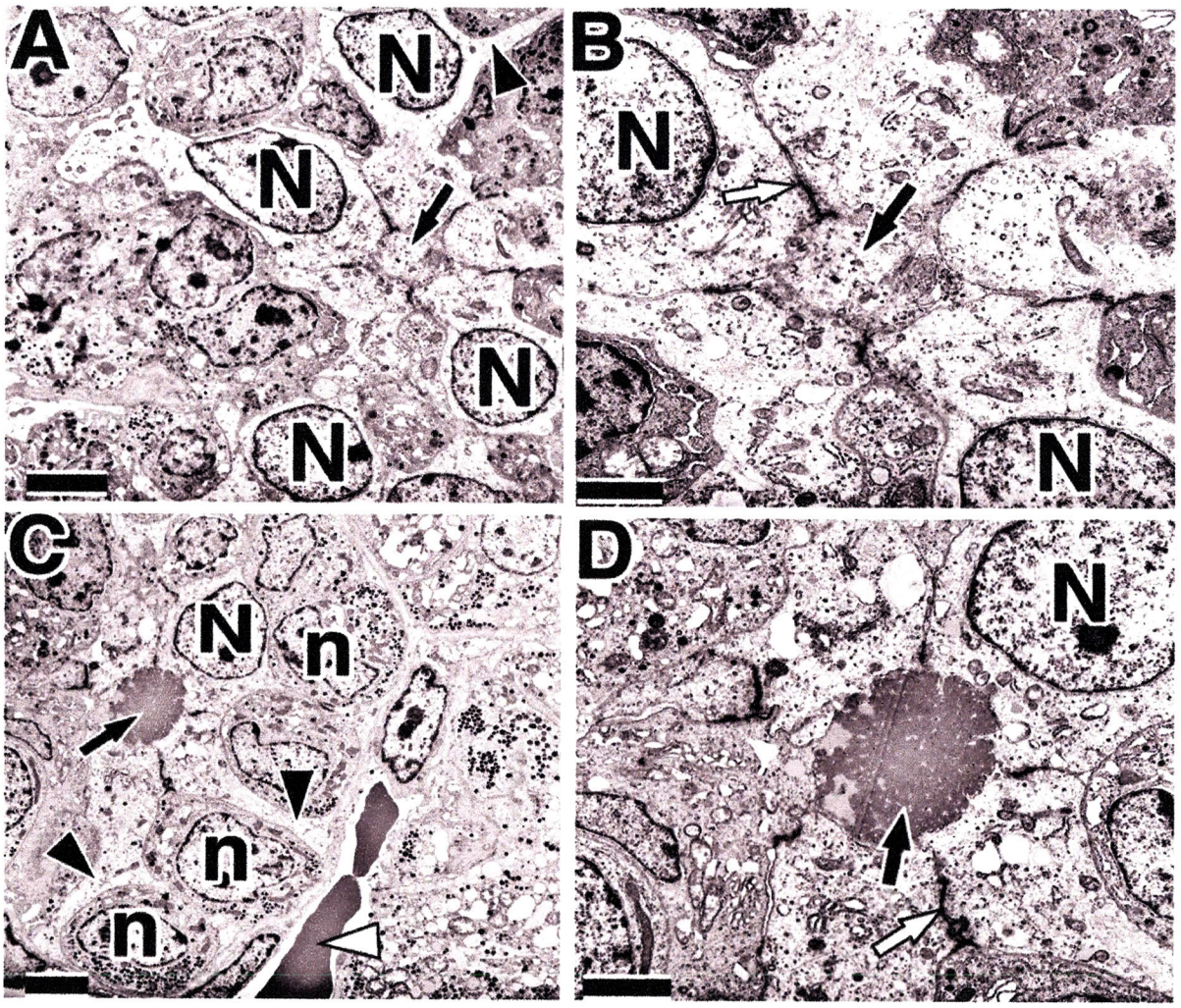

Fig. 5. Electron micrographs of round colloid-containing follicle. (A-D) Show round colloid-containing follicle (arrows) completely surrounded by FS cells (N). Higher magnification of the colloid in (A) is shown in (B) and that of (C) in (D) where homogenous and electron dense colloid is evident (arrows). FS cells extend cytoplasmic processes (arrowhead) to surround endocrine cells (n) and come in contact with blood capillaries (open arrowhead). Scale bar: A, C: 2μm ; B, D: 500nm

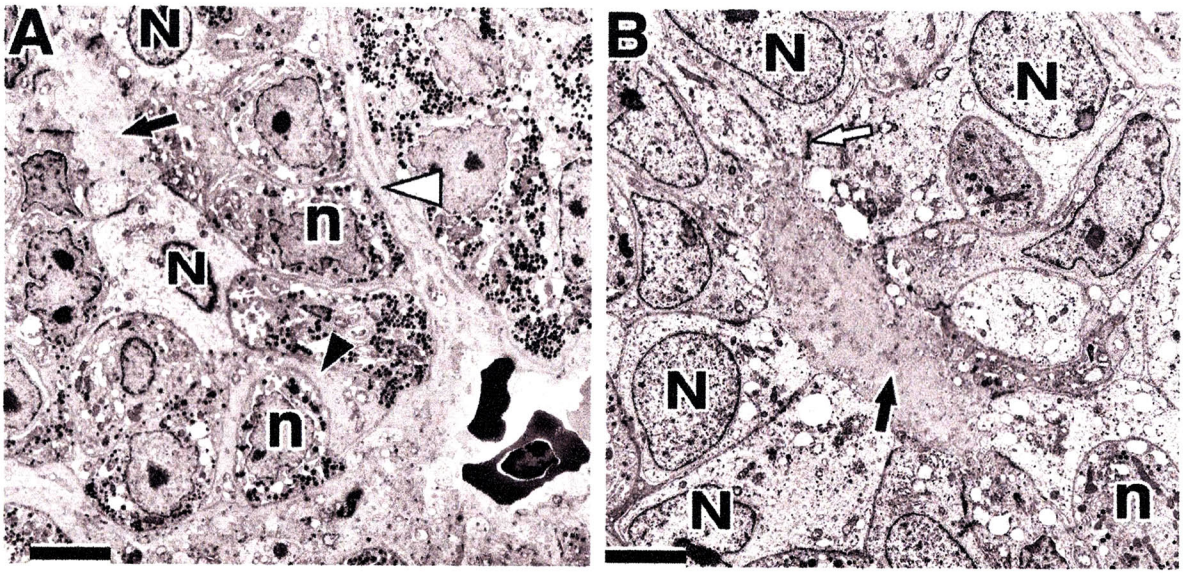

Fig. 6. Electron micrographs of irregular colloid-containing follicle. (A, B) micrographs illustrate irregular follicle containing translucent colloid (arrows). FS cells extend long processes (arrowhead) to surround endocrine cells (n). Open arrowhead marks the basement membrane and open arrow shows junction complex. Scale bar: 2μm

3.4 DISCUSSION

This study has confirmed the occurrence of colloid in follicular lumen in the pituitary gland of helmet guinea fowl. Presence of colloid in a 1-day-old bird indicates that their formation in this species of birds begin from prenatal life. This observation suggests that follicles in the anterior pituitary form basic morphological structure that is related to certain biological functions in neonatal life. Previous study on colloidal formation shows that first appearance of colloid containing follicles in the guinea pigs was recorded in 6month old animal (Kameda, 1991) and that they are rare and reduced in size in immature hamsters, hedgehogs and porcine (Spagnoli and Charipper, 1955; Ogawa et al. 1996; 1997). Our results are essentially consistent with those observed previously (Ciocca et al. 1984; Kameda 1990; 1991; Kubo et al. 1992) in which the number of colloidal accumulations in various animal species increased with age.

Most of well-developed colloid-containing follicles were completely surrounded by FS cells. However direct contact between colloid and granulated cells was also occasionally encountered. Such observation has also been reported in guinea pigs (Kameda 1990), suggesting that some granulated cells may be contributing to the formation of the colloid. In addition to the above observations, majority of FS cells were found to surround with their long cytoplasmic processes the actively dividing endocrine cells and aggregate with them to form a colloid-containing follicle. FS cells showed close association with colloid and capillaries. The relationship between FS cells and capillaries has also been reported in previous study (Soji et al. 1994).

FS cells are thought to initiate formation of colloid containing follicle and maintain their development. Colloid-containing follicles appeared to start within the cytoplasm of

FS cells by forming a predominant junction complex, lateral to the region where the follicle is going to be formed. Most of the follicles formed by FS cells were found surrounded by tight junctions. At some opening sites in the follicular lumen some droplets of colloid-like materials were visible. This observation suggests that the junctions were permeable to some substances. Such a phenomenon was also reported in previous studies (Vila-Porcile 1972; Allearts et al. 1990) in which tight junctions were found not complete but permeable to some materials.

From S-100 protein immunohistochemistry, the labeling of S-100 protein was first observed in the marginal cells layer of Rathke's residual pouch in the pars distalis within the caudal lobe near the junction with pars nervosa (posterolateroal region). The observation in which S-100 protein antibody staining not only the FS cells located in the parenchymal tissue but also the supramarginal cells in the Rathke's pouch was also reported in other studies (Correr and Motta, 1985). This study support the suggested possibility that FS cells may have developed from the rostral portion of the anterior wall of the Rathke's pouch, a structure derived from the oral cavity which is considered to be of ectodermal in origin.

CHAPTER IV

4.0 MOLECULAR CLONING, EXPRESSION PROFILE AND FUNCTIONAL IMPLICATIONS OF CLUSTERIN IN THE PITUITARY GLAND

4.1 INTRODUCTION

Clusterin is a highly conserved extracellular heterodimeric, disulfide-linked glycoprotein which is expressed in a wide variety of cell types (Jenne and Tschopp, 1992; French et al., 1993; Rosenberg and Silkensen, 1995; Mahon et al., 1999). Transcription generates a single full-length mRNA of about 1.6 kb which is translated on membrane-bound ribosome (Rowling and Freeman, 1993) and is targeted to the endoplasmic reticulum by a hydrophobic leader sequence for initial removal of signal peptide. Proteolytic cleavage into two α- and β-subunits of 40kDa each, dimer assembly and final glycosylation occur in the trans-Golgi network, resulting into an extracellular mature secreted protein, held together by a unique five disulfide bond motif (Wong et al., 1993; Yang et al., 2000; Jones and Jomary, 2002). However, it has been reported the existence of inactive, uncleaved, nonglycosylated clusterin isoform which is expressed in the cytoplasm and nucleus (Leskov et al., 2003; O'Sullivan et al., 2003).

Clusterin gene expression is induced in various biological processes including apoptosis, cellular injury, disease, regressing and involuting tissues (Leger et al., 1987; French et al., 1996; Viard et al., 1999). It is known to be suppressed by gonadal steroids in prostate, mammary gland and uterus (Bettuzzi et al., 1992; Wunsche et al., 1998) and by glucocorticoids in brain and kidney cells (Finch and May, 1995; Gutacker et al.,

1996). Secreted clusterin appears in almost all mammalian tissues and physiological fluids including plasma, milk, urine, cerebrospinal fluid and semen (Jenne and Tschopp, 1989; de Silva et al., 1990; Ghiso et al., 1993). Alternatively, secreted clusterin has been reported to occur as a monomeric intracellular protein in chicken and is not observed in serum (Mahon et al., 1999).

A series of observation support a link between clusterin and cell to cell interaction, sperm maturation, complement inhibition, lipid transport and membrane remodeling (Fritz et al., 1983; Jenne and Tschopp, 1989; de Silva et al., 1990; Tenniswood et al., 1992, Bailey and Griswold, 1999). Conversely clusterin shares chaperone functions with heat-shock proteins (Humphreys et al., 1999), which explains its ability to interact with a wide range of hydrophobic domains in other proteins (Jenne and Toschopp, 1992; Wilson and Easterbrook-Smith, 2000) and with partially folded denatured or stressed proteins to prevent their precipitation under stress conditions such as elevated temperature, reduction and oxidation (DeMattos et al., 2002; Poon et al., 2002; Carver et al., 2003). Moreover, clusterin has been reported to be associated with cell survival within tissues (French et al., 1994; Koch-Brandt and Morgans, 1996) and is considered to be induced under low or less toxic stimuli to exert cytoprotective effects on damaged or stressed cells. However, under high level of cytotoxic stress uncleaved, nonglycosylated disulfide linked isoforms of clusterin accumulate in the nucleus and hence contributes to cell death (Leskov et al., 2003; Trougakos and Gonos, 2004).

Clusterin gene has been isolated and sequenced in several species including human, rat, mouse and chicken (Wong et al., 1993; French et al., 1994; Jordan-Starck et al., 1994; Mahon et al., 1999). Sequence comparison among different mammalian species

revealed a high degree of conservation of about 70–85%. Clusterin has been proved to be the major glycoprotein that occurs in the colloid in the senescent porcine pituitary gland (Ogawa et al., 1997). Occurrence of colloid in the anterior pituitary has been studies in 43 species of birds and most numerous and largest colloid masses were observed in the guinea fowl (*Numida meleagris*) (Mamba et al., 1989). In this context, the guinea fowl appears to provide an excellent model for the study of physiologic role of clusterin in the pituitary gland and the relationship of clusterin accumulation in the colloid. The purpose of this study was therefore to investigate whether clusterin is implicated in the cellular processes leading to colloidal formation and accumulation in the pituitary gland. The full-length cDNA encoding guinea fowl clusterin was cloned and submitted to GenBank sequence database (accession number AY841278). Using this cDNA, RNA probes were prepared and used to examine the pattern of clusterin mRNA expression by in situ hybridization so as to determine the specific cell types synthesizing clusterin in anterior pituitary. Clusterin protein localization was investigated by immunohistochemistry and the relative level of clusterin mRNA expression in the pituitary and other related tissues was estimated by real-time quantitative RT-PCR.

4.2 MATERIALS AND METHODS

4.2.1 Tissues processing

All experiments were performed in accordance with the 1973 law concerning the protection and control of animals (guidelines for animal experimentation); Yamaguchi University. Twenty four adult (16months old) guinea fowls were used in this study. They were anesthetized with Nembutal (0.8ml/kg iv) and tissues were quickly excised. For cloning the guinea fowl clusterin cDNA, spleen tissue was used to isolate Poly $(A)^+$ RNA. Tissues including; brain, pituitary, lung, liver, spleen, pancreas, oviduct, ovary and testis were collected to determine clusterin mRNA expression levels by SYBR Green I real-time quantitative RT-PCR. Thirty (30) grams of each tissue was treated with 300ul RNA*later* RNA stabilization reagent and incubated overnight at 4°C followed by storage in –85°C until RNA extraction. For immunohistochemistry and *in situ* hybridization studies, a bird was perfused with Bouin's solution intracardiac following Nembutal anaesthesia. The pituitary gland was excised and fixed in Bouin's solution for 2 hours at 4°C before paraffin embedding.

4.2.2 Immunohistochemistry

The method for immunohistochemistry was essentially the same as described above. However, to detect S-100 protein and clusterin protein, the sections were incubated with polyclonal rabbit anti-cow S-100 protein (DAKO, Denmark, Code No Z0311) diluted 1:400 and monoclonal mouse anti-clusterin □-chain (human), clone 41D (Upstate Biotechnology, NY, USA, Catalog No 05-354) diluted 1:200. All the primary antibodies used were diluted in PBS.

4.2.3Polymerase chain reaction and cloning of guinea fowl clusterin full-length cDNA

Total RNA was extracted from spleen using a commercial kit (RNeasy® Protect mini kit, Qiagen Sciences, GmbH, Germany). RNA concentration was measured by absorption at 260nm. Poly(A)$^+$ RNA was purified with oligo(dT)-cellulose using oligotex-dT30 (super) mRNA purification kit (TaKaRa, Japan) and cDNA was synthesized with oligo (dT) [Not I-d$(T)_{18}$] primer by reverse transcription using First-strand cDNA synthesis kit (Amersham Biosciences, Uk). This cDNA was used to obtain the full-length guinea fowl clusterin by oligonucleotide-primed amplification by PCR (TaKaRa, Japan). Specific primers were chosen within the most conserved region of chicken, quail and human cDNAs (GenBank accession Number AF119370, X80760, BX648414). 5' end was amplified using forward primer chosen beyond start codon ATG 5'-GGGCTCTGGGACGGGACCATGGCGC-3'-coresponding to residues 1-25 and the reverse primer 5'-CTGCTGGATCGGGAGCAGCGGCA-3' corresponding to 490-512 residues of the Gallus clusterin cDNA. 3' end was amplified using forward primer 5'-GTGGGAGGAGTGCAAGCCCTGCC-3' corresponding to residues 333-355 and reverse primer was the oligo(dT) primer. The PCR fragments were subsequently cloned into the pGEM®-T easy Vector System II (Promega, USA) and both strands were sequenced by ABI 3100 DNA sequencer and analyzed by ABI software.

4.2.3 Estimation of clusterin mRNA levels in different tissues by real time RT-PCR

Preparation of Poly (A) $^+$ RNA from harvested tissues and synthesis of cDNA was done as describe above in cloning procedure. The cDNAs were amplified in real-time using the qPCR Master mix plus for SYBR Green I.

4.2.4 PCR Primers design

Specific primers were designed on guinea fowl clusterin cDNA using primer express software (Applied Biosciences). The combination of clusterin forward primer (5□ AGGAGACCAAGAGGAGGAAGGA 3') and clusterin reverse primer (5□ TCAAACACACCTGCATGCG 3') synthesized by Fasmac Co. LTD (Japan) allowed the amplification of a 145 bp fragment (nucleotides 230–374 in clusterin cDNA).

4.2.5 SYBR Green I real-time PCR.

Real-time PCR was performed in a GeneAmp 5700® Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR® Green I (10,000x concentration; Molecular probes, Eugene, OR, USA) as the detection format. Amplification was carried out in a total volume of 50 µl containing 0.5x SYBR® Green I, PCR buffer [50 mM KCl, 20 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2% glycerol and 0.2% dimethyl sulphoxide (DMSO)], 0.2 µM each primer, 0.2 mM dNTPs, 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 5 µl of 1 : 5 diluted cDNA. The cycling conditions consisted of an UNG pre-incubation step at 50 °C for 2 min, followed by a denaturation step at 95 °C for 10 min and then 40 cycles of amplification. Each cycle consisting of denaturation step for 15 s, 95 °C, followed by annealing for 1 min, 60 °C. A melting curve program was carried out from 60 to 95 °C for 20 min for each individual sample amplified with SYBR Green. The control cDNA was diluted from 1; 0.5; 0.25; 0.125 and 0.0625 and relative arbitrary quantities were defined. The threshold cycle (C_T), defined, as the cycle at which fluorescence rose above a defined baseline, was determined for each sample and control cDNA. A calibration curve was determined using the C_T values of the control cDNA samples, and the relative amount of unknown samples was deduced from this curve.

4.2.6 PCR data analysis

At the end of the PCR, the GeneAmp 5700® SDS software saved the results, allowing storage of the data for further analysis. Some initial cycles were considered as a baseline or background, in which no changes in fluorescence intensity occurred, and the level above this, at which increments in fluorescence became detectable, was the threshold (Th). The software also determined the cycle number when a reaction reached the threshold. This value, termed the 'cycle threshold' (C_T), appeared during the exponential phase of the PCR and was inversely proportional to the initial number of template molecules in the sample. A baseline from 1 to 13 and a threshold of 0.07 were used for all determinations.

4.2.7 In situ hybridization analysis

4.2.7.1 Preparation of RNA probes

The method for *in situ* hybridization was done using standard protocol (Sambrook and Russel, 2001). Briefly, riboprobes were prepared from *Numida meleagris* clusterin cDNA by PCR amplification of a 422bp fragment (nucleotides 91-512) using forward primer 5'-AGCGAGCTGAAACAGCTCTCG-3' and reverse primer 5' - TGCTGGATCGGGAGCAGCGGCA-3'. PCR products were ligated to pGEM-T easy vector (Promega, WI, USA) according to manufacturer's instructions. The cDNA insert was sequenced by ABI 3100 DNA sequencer, with T7 and SP6 sequencing primers. The purified plasmid was linearized, and the RNA probes were prepared and labeled with digoxigenin-UTP by *in vitro* transcription with SP6 and T7 RNA polymerase [digoxigenin RNA labeling kit (SP6/T7)] according to manufacturer's recommendations (Boehringer Mannheim).

4.2.7.2 Procedure for in situ hybridization

All steps prior to and during hybridization were conducted under RNase-free conditions. Sections (8 μm) were deparaffinized with xylene and rehydrated through descending ethanol concentrations (3 min each) and PBS. The sections were treated with proteinase K, 20 μg/ml in PBS, pH 7.4 at room temperature for 5 min. Then immersed in 0.2% (w/v) glycine in PBS (5 min) followed by hybridization in a humidified chamber overnight at 45 °C with prehybridization solution containing 10% dextran sulfate and 300 pg/μl of the digoxigenin-UTP labeled antisense or sense RNA probes. The slides were then washed three times for 10 min each time with 2x SSC and 0.5x SSC at 50 °C. After washing, slides were prepared for immunodetection by incubating them in 150 mM NaCl, 100 mM Tris, pH 7.5 (Buffer A) containing 3% normal goat serum and 1% bovine serum albumin for 30 min at room temperature. The sections were then exposed to anti-digoxigenin-Alkali phosphatase conjugate (1:500 dilution, Boehringer Mannheim) in the same buffer for 1 h at room temperature followed by extensive washing with Buffer A and then with Buffer B (100 mM NaCl, 100 mM Tris, pH 9.5, and 50 mM $MgCl_2$). The bound antibody was detected by incubating the slides with 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitroblue Tetrazolium (BCIP/NBT) substrate (Boehringer Mannheim), producing a blue precipitate reaction product. The reaction was stopped by washing sections with distilled water followed by mounting in entellan (MERCK, Darmstadt, Germany). Sections were examined with a Zeiss microscope.

4.3 RESULTS

4.3.1 Localization of clusterin protein within colloids and anterior pituitary cells

To identify the biochemical composition of colloid, pituitary sections were stained with antiserum to clusterin protein. Positive immunoreactivity was not only evident in the colloid but also in the cytoplasm of FS cells and endocrine cells. Clusterin positive FS cells were recognized by their special tendency to surround colloid masses and protrude long cytoplasmic processes to surround adjacent endocrine cells (Figs. 7A-D). However, not all FS cells surrounding colloid showed localization of clusterin in their cytoplasm. Following these findings, interest was developed to investigate the expression pattern of clusterin mRNA in specific cell types in the pituitary and determine its site of synthesis. The full length cDNA of guinea fowl clusterin was cloned and analyzed. Using this cDNA, RNA probes were prepared to be used for in situ hybridization studies.

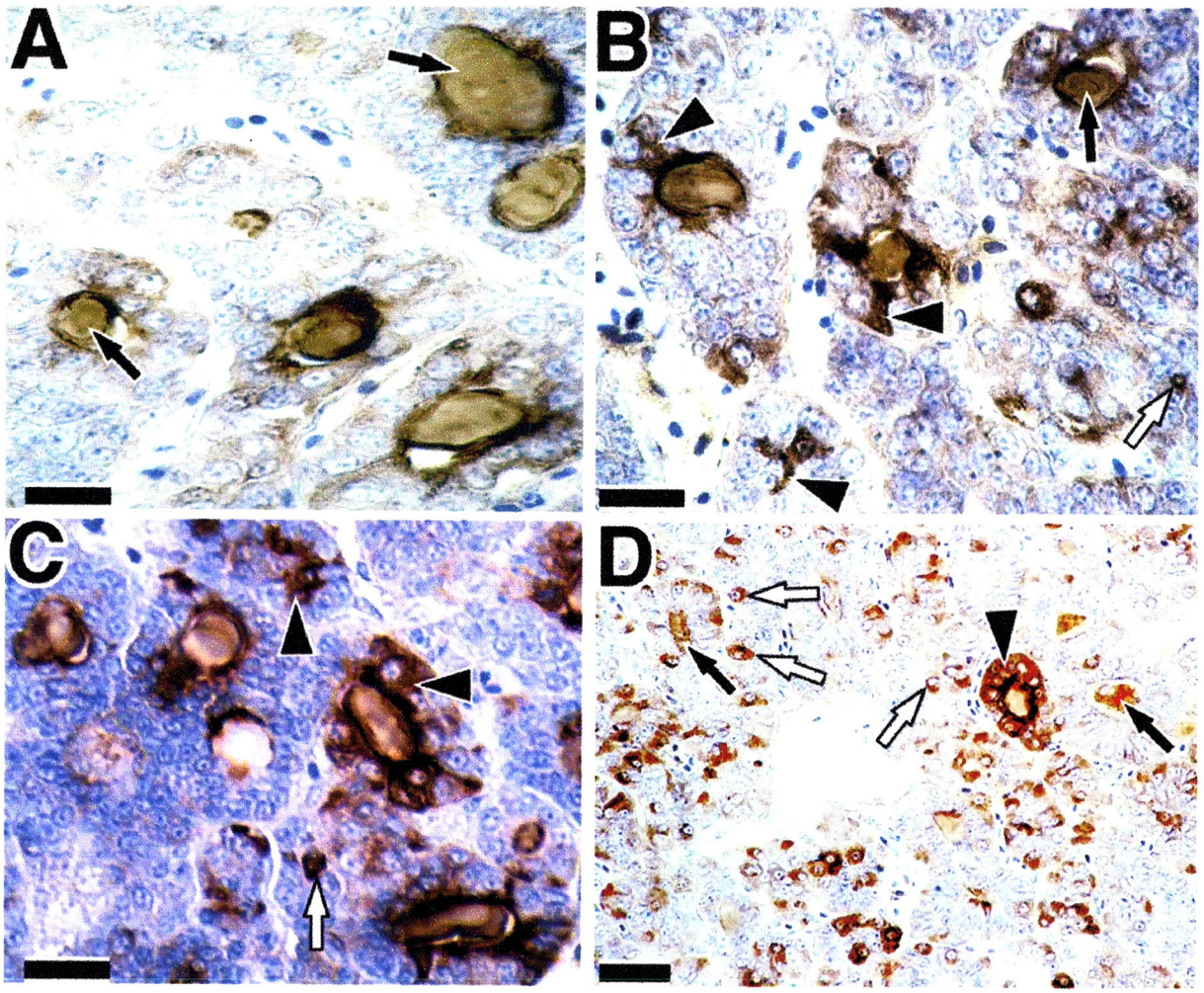

Fig. 7 Positive immunohistochemistry of clusterin protein in anterior pituitary cells and the colloid. (A-D) Show positive immunoreactivity of clusterin protein in the colloid (arrow), endocrine cells (open arrows) and FS cells (arrowheads). FS cells are recognized by their special characteristics to surround colloidal materials they produce and to protrude long cytoplasmic processes to surround adjacent endocrine cells. Scale bar: A, B, C: 70μm; D: 100μm

4.3.2 Isolation of the cDNA encoding for guinea fowl clusterin

A full-length guinea fowl clusterin cDNA was isolated from spleen. The deduced nucleotide sequence contains an open reading frame of 1344 nucleotides from the first translation initiation codon "ATG" at position 19 to translation stop codon "TAG" at positision 1362 and encodes a protein of 448 amino acid residues. Location of the first translation initiation codon (ATG) in the context; ACCATGG, precisely conforms to both the Kozak (1987) and Cavener and Ray (1991) consensus sequence required for translation initiation. The guinea fowl clusterin has a putative signal sequence consisting of 20 amino acids generating an amino terminal leucine in the mature protein (Fig. 8). The amino terminus of the guinea fowl located at the same position with quail, chicken and human clusterin has been proposed as the glycine 4 residues upstream of this leucine (Mahon et al., 1999).

Analysis of the deduced amino acids by multiple sequence alignment for guinea fowl with those of chicken (*Gallus gallus*), quail (*Coturnix coturnix*) and human (*Homo sapiens*) homology (GenBank accession Number AF119370, X80760, BX648414) revealed 97, 95 and 45% sequence identity respectively. All 10 cysteine residues are conserved in the four species as well as four of the seven consensus sequences for potential N-linked glycosylation in the human protein. Guinea fowl has two additional consensus sequences for N-linked glycosylation that are absent in human but conserved in chicken and quail clusterin. The putative heparin binding domains and amphipathic α-helical regions predicted from the human sequence (de Silva et al., 1990) are not strictly conserved. A potential nuclear localization signal (LEEAKKKK) in human clusterin and proposed to target the truncated form of clusterin protein to the nucleus (Reddy et al., 1996) is present in the guinea fowl clusterin in the context (LEETKRRK)

identical in that region to chicken and quail clusterin. Three amino acid residues corresponding to codons 142, 295 and 398 are conserved in the guinea fowl, identical to human and quail clusterin but not in the chicken clusterin (Fig. 9).

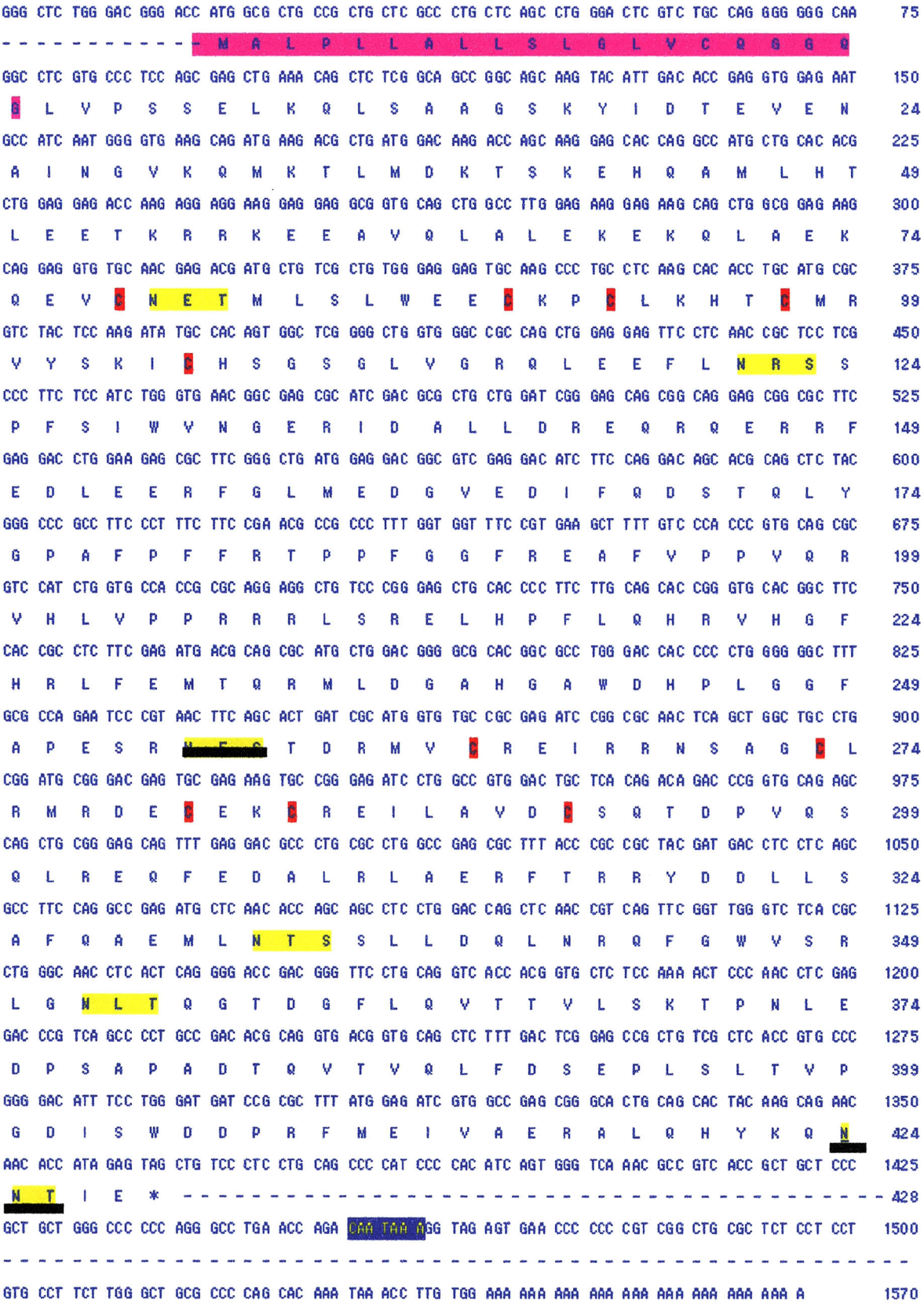

Fig. 8. For legend refer to page 49

Fig. 8. Nucleotide and deduced amino acid sequences of the cDNA for guinea fowl clusterin. The amino acid sequence is shown in single-letter code beneath the cDNA sequence. Untranslated nucleotide sequences are underscored by dotted line. Numbering of the amino acids refers to the mature protein. Amino acid residues 1-20 (marked pink) represent the putative signal peptides. Ten conserved cysteines are indicated by C-red marked letters. The six potential N-linked glycosylation sites are marked yellow where bold-underlined indicates absence in human clusterin. Asterisk represents termination codon and polyadenylation signal is shown in blue marked letters. Guinea fowl clusterin cDNA sequence is available in GenBank sequence database under accession number AY841278.

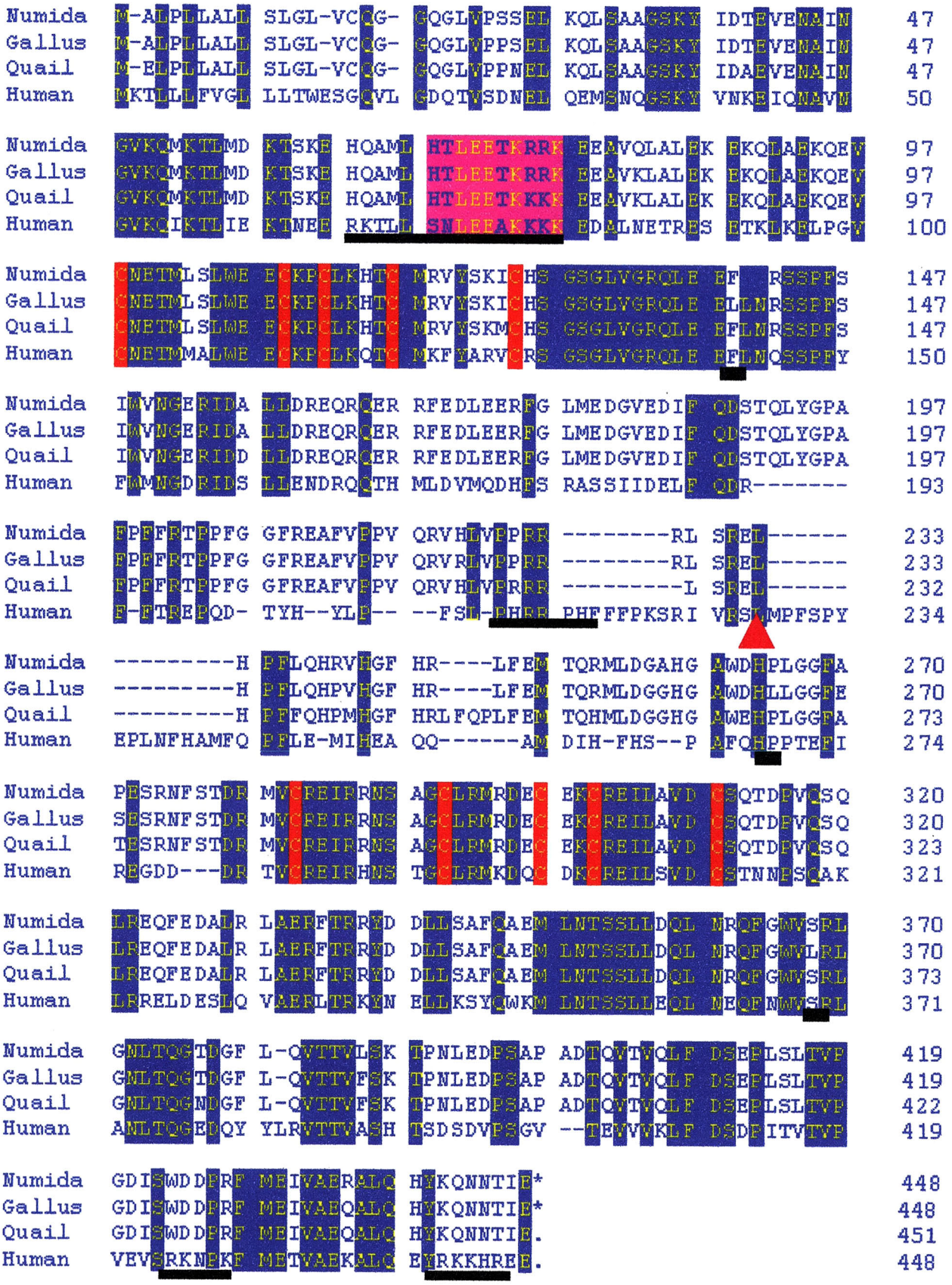

Fig. 9. For legend refer to page 51

Fig. 9. Comparison of the clusterin protein sequences of guinea fowl, chicken, quail and human. Identical amino acid residues in all four species are shown in blue highlight while black squire boxes underneath indicate not conserved in chicken. Numbering starts with the respective translation initiator methionines. Gaps indicated by dashes were introduced to optimize the alignment. The ten conserved cysteines are marked red. The reported four potential heparin binding sites in the human clusterin sequence (de Silva *et al.* 1990) are underlined. The putative nuclear localization sequences (Reddy *et al.* 1996) are shown by pink marked letters and the red upward arrowhead indicates the predicted cleavage site producing the α- and β-subunits in human clusterin (de Silva *et al.* 1990).

4.3.3 Relative quantification of clusterin mRN A obtained by real-time RT-PCR

4.3.3 Relative quantification of clusterin mRN A obtained by real-time RT-PCR

Relative quantification results showed that Clusterin mRNA levels were highest in the Testis (2.65 copies/reaction) and lowest in the pancreas (0.051 copies/reaction). Result summary is presented in Table 3.

Table 3: Quantification of clusterin mRNA by real-time RT-PCR in tissues collected from 16 months old helmeted guinea fowl.

S/N	Tissue	mRNA copies/ml
1	Brain	1.56
2	Pituitary	0.699
3	Lung	0.267
4	Spleen	0.387
5	Liver	0.923
6	Pancreas	0.0502
7	Oviduct	0.761
8	Ovary	1.9
9	Testis	2.65

4.3.4 Expression of clusterin mRNA in the anterior pituitary cells

The expression of clusterin mRNA was detected in the cytoplasm of endocrine cells and FS cells by the antisense digoxigenin-UTP labeled RNA probe. The distribution of hybridization signals did not appear to be associated with any particular cell type. (Fig. 10A-C). The labeled sense probe showed no clusterin mRNA signals in the sections (Fig. 10D). However, cells (FS cells) surrounding colloid masses intensely expressed clusterin mRNA (Fig. 11A-E). From EM observation, fully developed colloid-containing follickes are frequently observed to be completely surrounded by FS cells. The observation of clusterin mRNA signal around colloids signifies the ability of FS cells to synthesize clusterin.

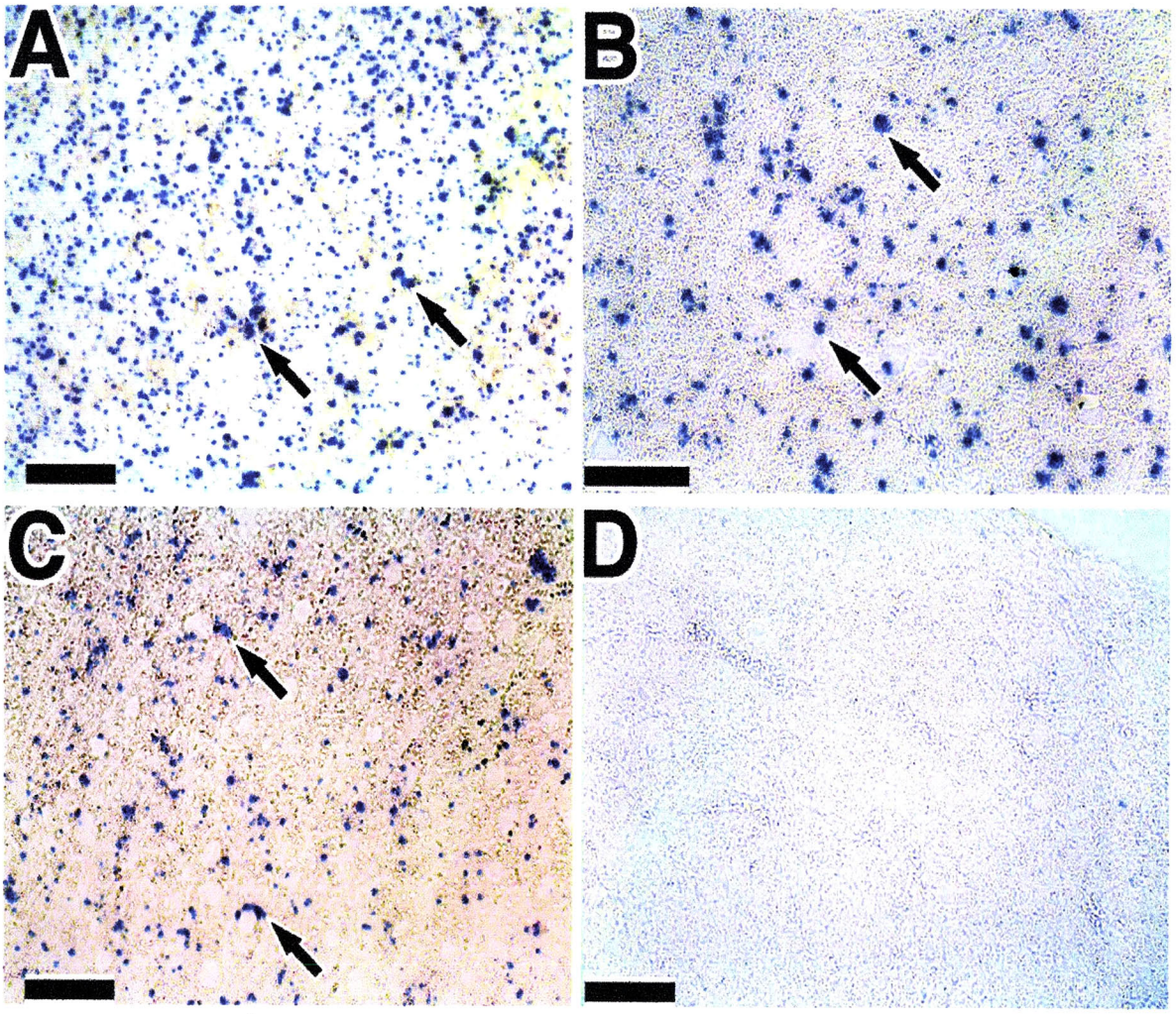

Fig. 10. Expression of clusterin mRNA in the anterior pituitary. Clusterin transcripts signal is observed in many cells in the anterior pituitary (arrows) as detected by in situ hybridization of digoxigenin-UTP labeled clusterin antisense RNA probe (A-C). Transcript signals do not appear to be associated with any particular cell type and are not observed in sections incubated with digoxigenin-UTP labeled sense probe (d). Scale bar: A, C, D: 100μm; B: 70μm

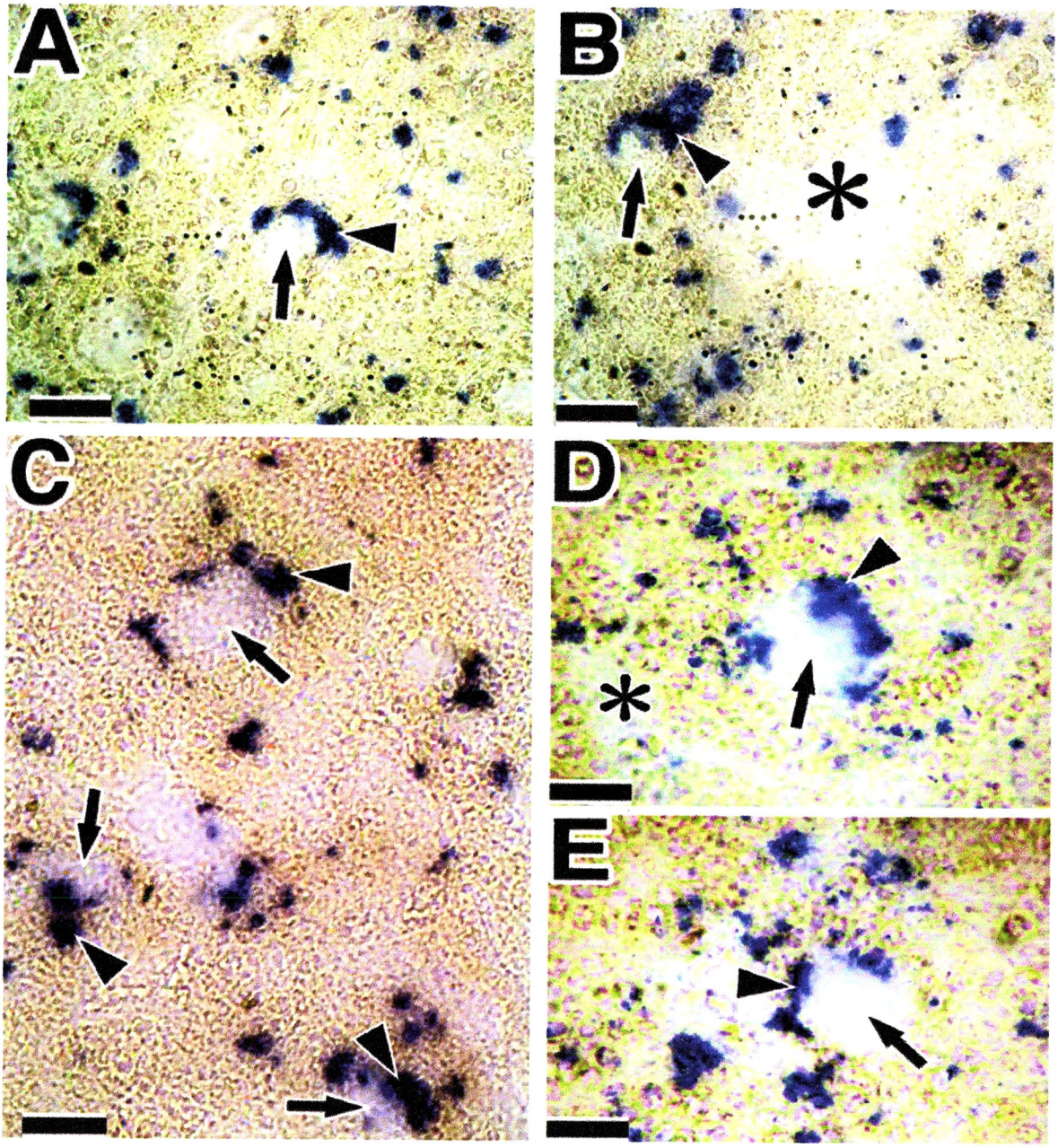

Fig. 11A-E. Expression of clusterin mRNA in FS cells surrounding colloidal accumulations. Clusterin transcript signals detected by in situ hybridization are observed to be expressed in FS cells (arrowheads) surrounding colloidal accumulations (arrows). Asterisks show the site of blood capillaries. Scale bar: A, B: 70μm; C, D, E: 50μm

4.3.5 Simultaneous localization of S-100 protein and clusterin mRNA hybridization signals in the same FS cell

To confirm the expression of clusterin mRNA in FS cells, after *in situ* hybridization the sections were immunostained by S-100 antibody, a specific marker for FS cells. The brown colour is DAB reaction product for S-100 immunostaining) and the blue colour is the formazan dye product of BCIP/NBT reaction for clusterin mRNA. Brown and blue colours enabled visualization of S-100 protein and clusterin mRNA in the same FS cell in single section. Apart from FS cells surrounding colloid containing follicles, many other isolated FS cells were found to be associated with clusterin transcript signals (Fig. 12A, B).

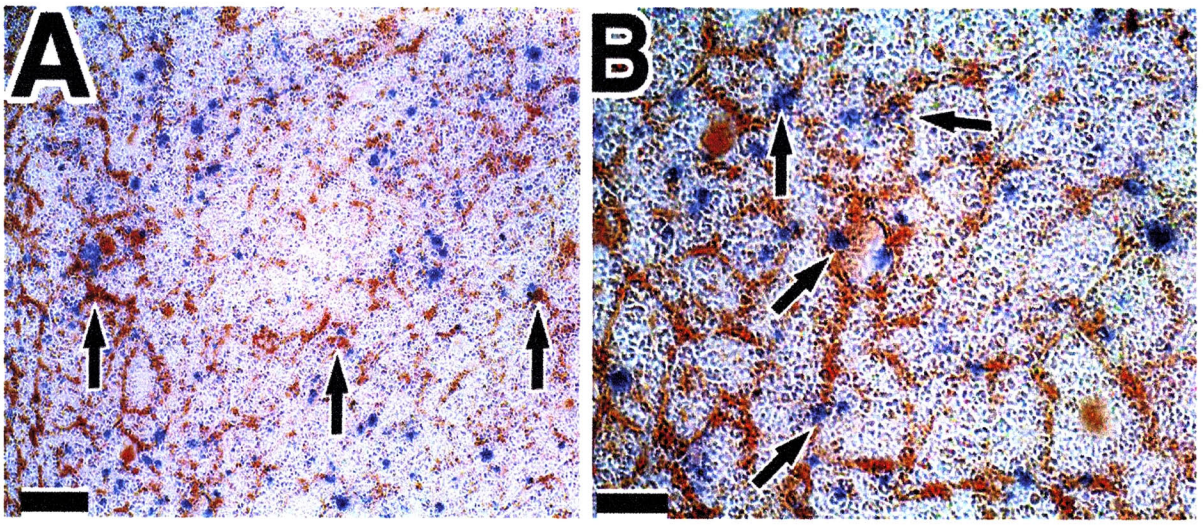

Fig. 12A, B. Co-localization of clusterin mRNA hybridization signals and S-100 protein in the same FS cell. Detection of clusterin mRNA hybridization signals and S-100 protein in the same cell by in situ hybridization and immunohistochemistry of S-100 protein respectively suggests that clusterin mRNA is expressed in FS cells (arrows). S-100 protein is a known marker of FS cells in the anterior pituitary. Scale bar: A: 100μm; B: 50μm.

4.4 DISCUSSION

Sequence analysis of the guinea fowl clusterin cDNA and comparison with the available avian clusterin cDNA sequences (chicken and quail) revealed a high degree of sequence homology and conservation of the predicted structural features of the proteins among the three species (Fig. 1; 2). Clusterin protein sequence around the known cleavage site in human clusterin (RIVR-cleavage-SLM) is different from that in the guinea fowl but identical to quail and chicken clusterin (RLSR-cleavage-ELH). However all three clusterin sequences in the birds appear to conform to cleavage site requirements for furin (R*XX*R) (Molloy et al., 1992). These findings suggest that there are evolutionary changes in clusterin among different species from the production of a single-chain protein in birds as reported in chicken (Mahon et al., 1999), to that of heterodimeric secreted form in mammals.

The pattern of clusterin mRNA expression observed in various tissues in the guinea fowl was similar to that reported in other vertebrates (Fleming et al., 1992; French et al., 1993; Wong et al., 1993; Mahon et al., 1999) in which high levels ware observed in the gonads. In the guinea fowl, clusterin mRNA was particularly abundant in the testis, ovary, brain and spleen and at lower levels in the liver, oviduct, pituitary, pancreas and lungs. These results suggest that the biological function of clusterin is well conserved in all species.

Our findings on clusterin mRNA expression in anterior pituitary of guinea fowl coincide well with published reports on clusterin expression in the pituitary gland of bovine and sheep (Laslop et al., 1993; Fleming et al., 1999) as well as clusterin protein localization in the endocrine cells (Inoue et al., 2002). The only exception to this is that

we have observed clusterin mRNA and protein in FS cells in the guinea fowl. This phenomenon was not observed in other animal species. In this meaning, this paper is the first confirmation of clusterin accumulation in the pituitary gland. Moreover, clusterin was not observed in all endocrine cells and FS cells at the same time, this could be associated with hormonal status of the animal and function of a cell at that time. A normal cell responds to stress stimuli or damage 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.

Colloid masses are observed to be completely surrounded by FS cells (Ogawa et al., 1996). We have confirmed this observation by immunohistochemistry and electron microscopy (Unpublished data). In this context, FS cells are considered to be involved in colloid formation (Kagayama, 1965; Fukuda, 1973). FS cells are also observed to extend long cytoplasmic processes to surround adjacent endocrine and produce many substances that influence adjacent 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). Consistent with previous studies, we have observed clusterin mRNA signals and protein in FS cells around colloid masses and surrounding endocrine cells. This observation suggests that FS cells regulate the function of endocrine cells. Colocalization of S-100 protein and clusterin mRNA in the same FS cell confirmed further the ability of these cells to synthesize clusterin.

Previous studies on cells death show that clusterin appears before apoptosis in an apparent attempt by a cell to protect itself from cells death, although these cells for some reasons undergo apoptosis (Viard et al., 1999). Results obtained in this study are consistent with the hypothesis that endocrine cells produce clusterin before apoptosis to exert cytoprotective effect. Degenerating endocrine cells are phagocytosed by FS cells and digested by their lysosomal enzymes. The phagocytotic activity of these cells has been clearly demonstrated in the pituitary gland of estrogen-deficient rats, which is known to induce apoptosis of prolactin cells (Drewett et al., 1993). In the FS cells, clusterin interacts and aggregates with molecules, proteins and peptides resulting from their digestion and subsequently become stored in the follicular lumen as colloid. This hypothesis is supported by the observation of clusterin in endocrine cells, FS cells and in the colloid. Involvement of clusterin in clearance of cellular debris is achieved by the presence of putative amphipathic α-helices that mediate hydrophobic interactions with numerous types of molecules (Jenne and Tschopp, 1989; Tsuruta et al., 1990).

CHAPTER V

5.0 PHAGOCYTOTIC REMOVAL OF APOPTOTIC ENDOCRINE CELLS BY FOLLICULOSTELLATE CELLS AND ITS FUNCTIONAL IMPLICATIONS IN CLUSTERIN ACCUMULATION IN PITUITARY COLLOIDS

5.1 INTRODUCTION

Periodic acid-Schiff reactive colloids have ben observed in the pituitary gland of various animals such as bat, cattle, guinea pigs, viscacha and human (Bassett, 1951; Anthony and Gustafson, 1984; Ciocca et al., 1984; Kameda, 1990; Mohamed et al., 2000). It is also known that these colloids increase in number with age (Kameda, 1991). Various theories have been proposed regarding the mode of colloid formation. Repeated observation of cell fragments within colloid suggests that colloidal materials arise as a by-product of cellular degradation (Horvath et al., 1974; Ciocca and Gonzales, 1978). An alternative hypothesis suggests that colloid is derived at least in part from interstitial fluid of the pars distalis (Villa-Porcile, 1972; Correr and Motta, 1981). Studies show that colloids are completely surrounded by folliculostellate cells (FS cells) and therefore it is generally accepted that the major part of colloid is produced by FS cells (Kagayama, 1965; Fukuda, 1973; Kameda, 1991). However there is no thorough and systematic analysis to demonstrate the origin of pituitary colloids and the mode of their secretion *in vivo*.

Apart from colloid secretion, FS cells are reported to exhibit phagocytosis by acting as scavenger cells of the anterior pituitary (Inoue et al., 2002). The phagocytotic function of these cells has been clearly demonstrated in the pituitary gland of estrogen-deficient

rats, which is known to induce apoptosis of prolactin cells (Drewett et al., 1993). FS cells are also observed to extend long cytoplasmic processes and produce many substances 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) that influence adjacent endocrine cells.

Colloids have further been purified and analyzed by cytochemistry and found that clusterin is the major glycoprotein in them (Ogawa et al., 1997). Clusterin is shown to have a relationship with apoptosis. It is associated with cell survival within tissues (French et al., 1994, Koch-Brand and Morgan, 1996) and is induced under low toxic stimuli to exert cytoprotective effects on damaged or stressed cells. However, under high level of cytotoxic stress, uncleaved, nonglycosylated clusterin isoforms accumulate in the nucleus and contribute to cell death (Leskov et al., 2003, Trougakos and Gonos, 2004). Activation of apoptosis is under the control of a network of inter-related signals that originate both from the intracellular and extracellular milieu. Apoptotic cells are morphologically characterized by cell shrinkage, cytoplasmic blebbing, chromatin condensation and formation of membrane-bound fragments of the nucleus called apoptotic bodies (Wyllie et al., 1984). In the anterior pituitary, there is high rate of cell death process by apoptosis. On average, each anterior pituitary cell undergoes either mitosis or apoptosis once every 63 days depending on the endocrine status of the animal (Haggi et al., 1986; Oishi et al., 1993; Nolan et al., 1998; Yin and Arita, 2000). The equilibrium of such processes is maintained by a continuous renewal of cells and therefore the pituitary gland exhibits apparent cyclic pattern of cell turnover.

While the occurrence of colloidal accumulations in anterior pituitary has been reported in several animal species, much less is known on their origin, composition and biological importance. The phagocytotic activity of FS cells and the relationship of clusterin accumulation in the colloid are also poorly understood. The purpose of this study was therefore to examine by light and electron microscopy, apoptosis and phagocytotic activity of FS cells *in vivo* and assess its functional implications in colloidal accumulation in the anterior pituitary in the guinea fowl.

5.2 MATERIALS AND METHODS

5.2.1 Tissue preparation

All experiments were performed in accordance with the 1973 law concerning the protection and control of animals (guidelines for animal experimentation) Yamaguchi University. Paraffin embedded pituitary specimens were prepared from 24 adult guinea fowls aged 16 months. They were anesthetized with Nembutal [pentobarbital] (0.8ml/kg iv) and pituitaries were quickly excised and processed for light or electron microscopy. For Hematoxylin-Eosin (H&E) and periodic acid-Schiff (PAS) reaction, a pituitary gland was sectioned in sagittal plane and fixed either in Bouin's solution or 10% formalin before embedding in paraffin. Pituitary tissues were sectioned in horizontal plane at a thickness of 4μm and stained with H&E and PAS reaction. Morphological studies and cross section examination were performed using a Zeiss microscope. For immunohistochemistry and *in situ* hybridization studies, a bird was perfused with Bouin's solution intracardiac following Nembutal anaesthesia and the collected pituitary glands were fixed in Bouin's solution for 2 hours at 4°C before paraffin embedding.

5.2.2 Immunohistochemistry

To detect S-100 protein, single stranded DNA (ssDNA) and clusterin protein, the sections were incubated with polyclonal rabbit anti-cow S-100 protein (DAKO, Denmark, Code No Z0311) diluted 1:400, polyclonal rabbit anti-ssDNA antibody (Dako ytomation, Code No A4506) diluted 1:100, and monoclonal mouse anti-clusterin □-chain (human), clone 41D (Upstate Biotechnology, NY, USA, Catalog No 05-354) diluted 1:200, respectively. All the primary antibodies were diluted in PBS and applied to sections for 24 h in a dark, humid chamber at 4 °C. The immunohistochemical procedures were essentially the same as described previously.

5.2.3 In situ hybridization

Probes for *in situ* hybridization were prepared from *Numida meleagris* clusterin cDNA (GenBank database accession number AY841278) by PCR amplification of a 422bp fragment (nucleotides 91-512) using forward primer 5'-AGCGAGCTGAAACAGCTCTCG-3' and reverse primer 5' - TGCTGGATCGGGAGCAGCGGCA-3'. PCR products were ligated to pGEM-T easy vector (Promega, WI, USA) according to manufacturer's instructions. The cDNA insert was sequenced by ABI 3100 DNA sequencer, with T7 and SP6 sequencing primers. The purified plasmid was linearized, and the RNA probes were prepared and labeled with digoxigenin-UTP by *in vitro* transcription with SP6 and T7 RNA polymerase [digoxigenin RNA labeling kit (SP6/T7)] according to manufacturer's recommendations (Boehringer Mannheim). All steps for *in situ* hybridization was done as described previously.

5.2.4 Electron microscopy

The method for electron microscopy was done as described in experiment I. Briefly pituitaries were rapidly excised and sliced into 1mm slabs in a few drops of pre-fixative solution (2% Paraformaldehyde, 2.5%Glutaraldehyde in 0.1M Sodium Cacodylate buffer, pH7.4 with 0.2mM $CaCl_2$). The tissues were then pre-fixed in the same pre-fixative solution for 90 minutes at 4°C with gentle shaking. After a rinse in 0.1M Cacodylate buffer, pH7.4, the tissues were post-fixed in 2%Osmium tetroxide for 90 min at 4°C followed by dehydration and embedding in epoxy resin. Ultra thin sections of approximately 70nm thick were cut, mounted on grids and stained with 2% uranyl acetate for 20 min and Lead citrate for 7 min. Specimens were finally examined at 80kV in transmission electron microscope (Hitachi H-7600 TEM Version 3.01).

5.3 RESULTS

5.3.1 Routine histological examination findings

Anterior pituitary sections were stained with PAS reaction and H&E. PAS positive colloids were observed as described in experiment I. On H&E, some colloidal materials were homogenous and densely stained with hematoxylin while others were scanty and translucent with low affinity to staining dye. Some cells particularly those located adjacent to colloid masses showed very dense staining of chromatin by haematoxylin. Condensation and reduction in size of the cells were also observed. (Fig. 13A-D).

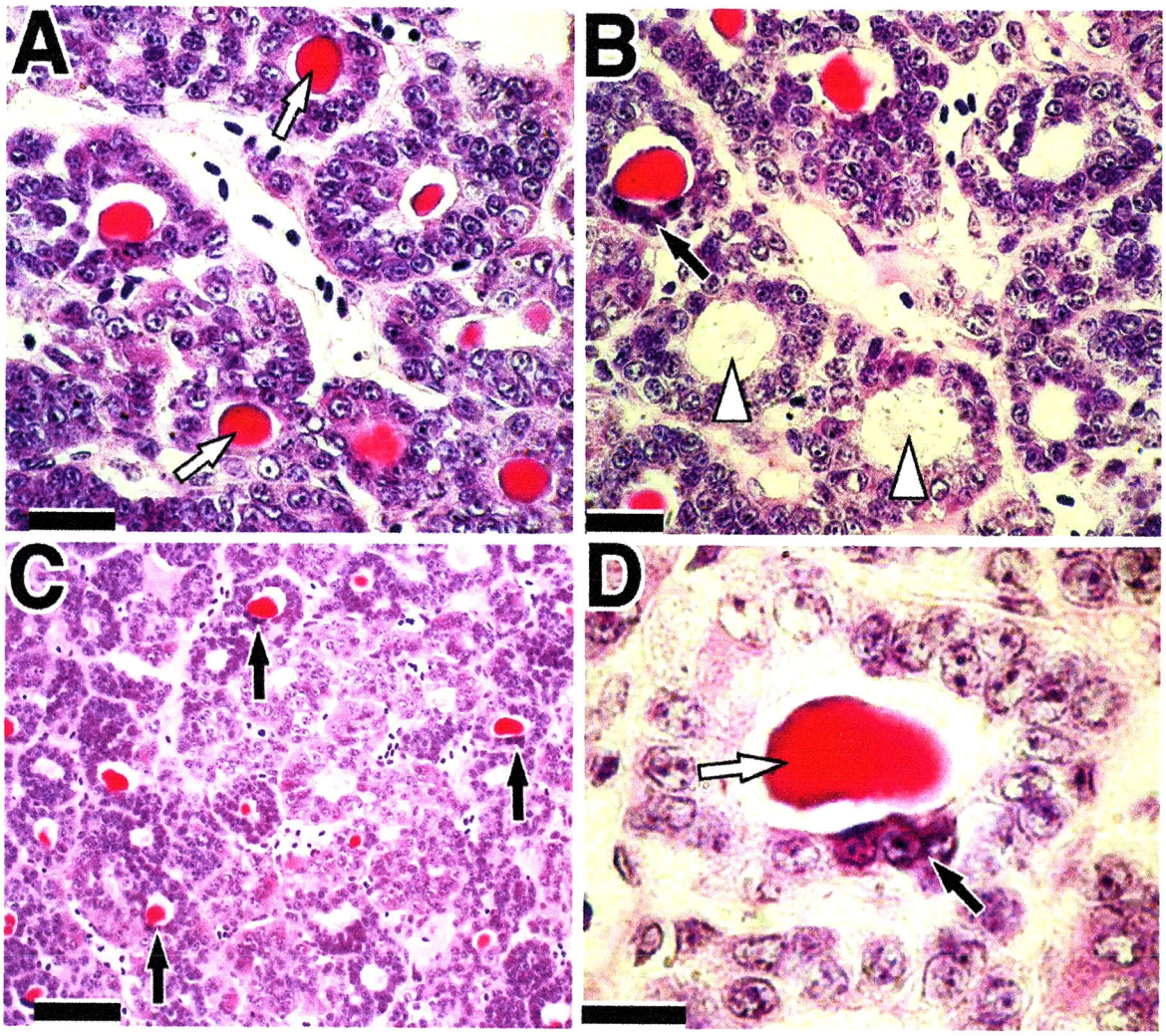

Fig. 13. Apoptotic cells revealed by H&E staining in the anterior pituitary. Extracellular accumulations of colloid (open arrows) are observed at the centre of cell clusters surrounded by a thin connective tissue capsule (A). Two kinds of colloids according to staining intensity are observed: large, homogenous, densely stained colloid (open arrows) and scanty, translucent colloid with low affinity to staining dye (open arrowhead). Very dense staining of chromatin by haematoxylin (arrows) is seen in endocrine cells bordering colloid masses (B-D). Chromatin condensation, a characteristic of apoptosis is evident in the cells as pyknosis. Higher magnification of (C) is shown in (D). Scale bar: A, B: 50μm; C: 100μm; D: 30μm.

5.3.2 Immunohistochemical observations

In experiment II clusterin was observed to be localized in the colloids, endocrine cells and FS cells. Clusterin is also reported to have a relationship with apoptosis. In the anterior pituitary, apoptosis was analyzed by immunohistochemistry of ssDNA. Randomly distributed ssDNA positive cells in early and late stages of apoptosis were observed as light and dark brown deposits of DAB. Some dark nuclei were observed localized adjacent to colloid masses (Fig. 14A-D).

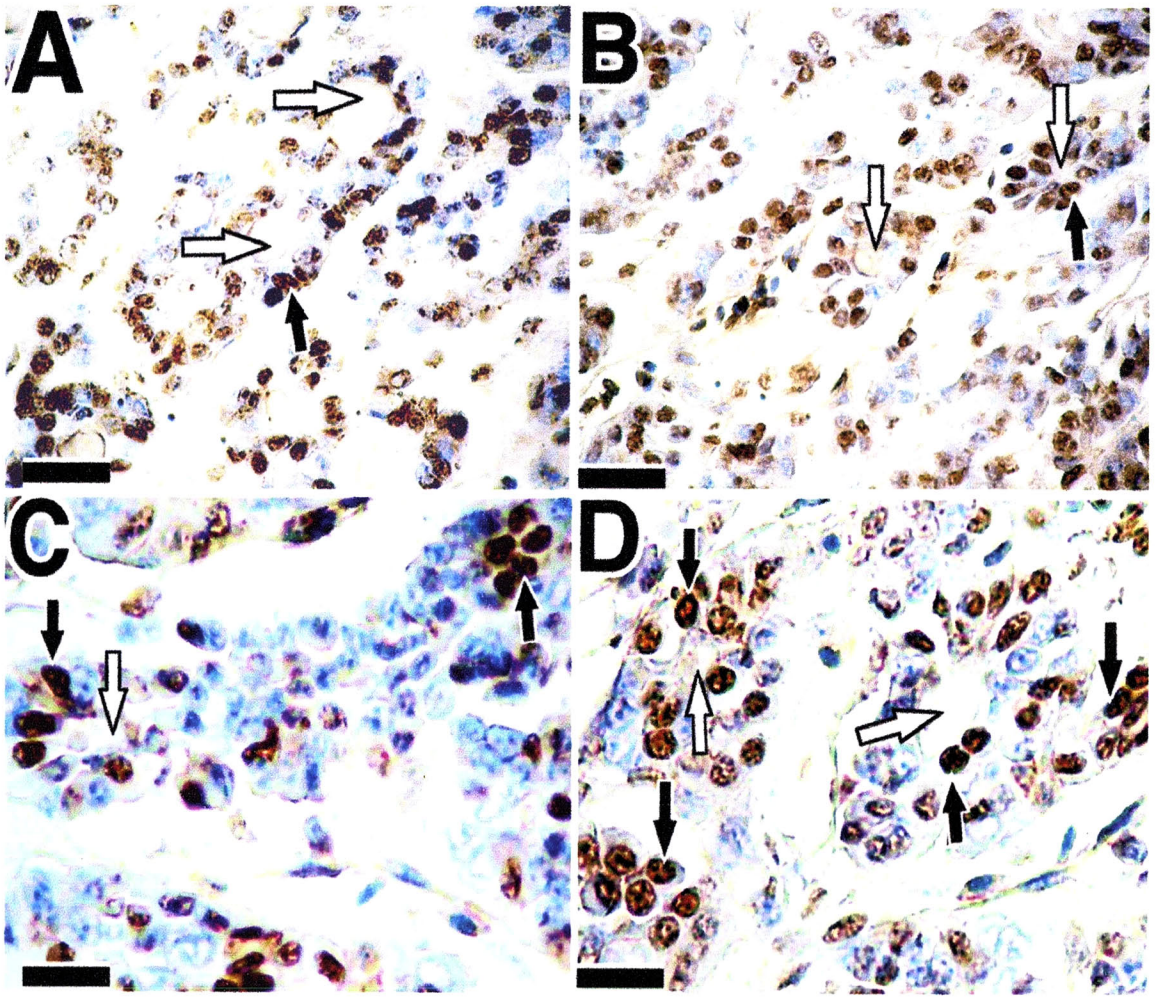

Fig. 14A-D. Immunohistochemical analysis of apoptosis in the anterior pituitary by anti-ssDNA polyclonal antibody. Brown nuclei (arrows) are apoptotic cells positive for anti-ssDNA antibody. Early and late stages of apoptosis are manifested by respective light and dark brown deposit of the peroxidase chromogen DAB. Majority of apoptotic cells are observed to be adjacent to colloid masses (open arrows). Light blue nuclei are non-apoptotic cells, counterstained with hematoxylin. Scale bar: 70μm.

In order to asses whether clusterin mRNA expression and apoptosis within anterior pituitary concerns the same cell, in situ hybridization of clusterin mRNA was performed on pituitary sections, followed by immunohistochemistry of ssDNA on the same sections. The simultaneous expression showed that locations of clusterin mRNA expressing cells and that of apoptotic cells were mutually exclusive and thus that clusterin mRNA is not expressed by apoptotic cells but in neighbouring surviving cells (Fig. 15A, B).

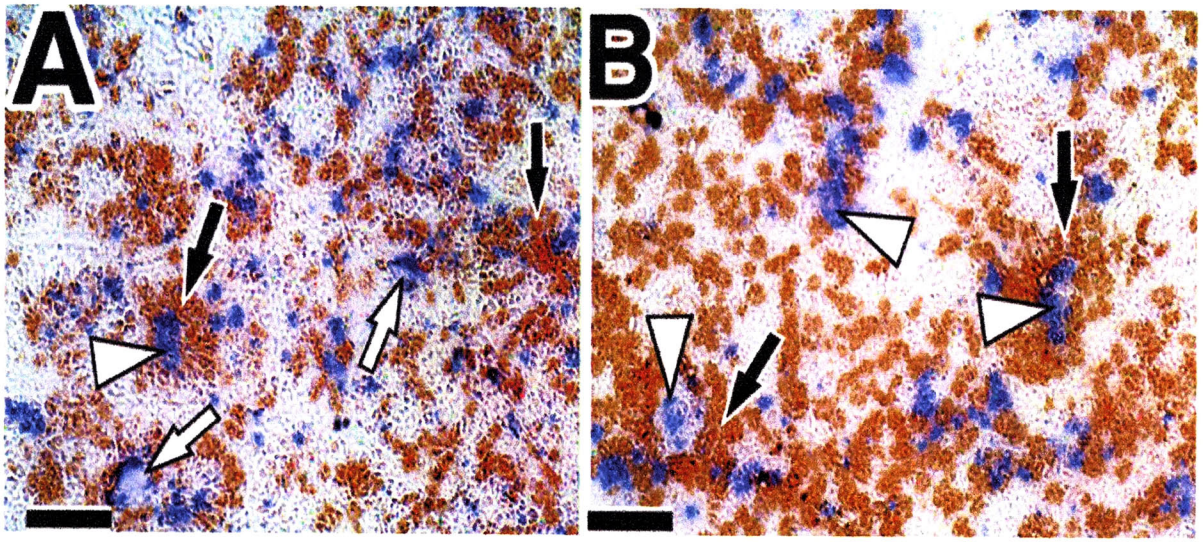

Fig. 15A-B. Simultaneous labeling of clusterin mRNA by in situ hybridization and ssDNA by immunohistochemistry. Expression of clusterin mRNA (open arrowheads) is seen as a blue reaction product produced from an alkaline phosphatase reaction with BCIP/NBT, where as ssDNA in apoptotic cells (arrows) is evident as brown reaction product resulting from a DAB peroxidase reaction. Almost all cells expressing clusterin mRNA are observed to be close to or surrounded by ssDNA positive cells. Open arrowheads show colloids. Scale bar: 70μm.

5.3.3 Observed ultrastructural features

Electron microscopic observation generally agreed well with results obtained by light microscopy. Colloid appeared completely surrounded by FS cells. Typical FS cells were recognized by electron microscope by their lack of cytoplasmic granules. Some morphologically intact endocrine cells were frequently observed to be engulfed by FS cells (Fig. 16A-D). Some were characterized by cellular shrinkage, nuclear condensation and formation of membrane-bound fragments (apoptotic bodies). Mitochondria and lysosomes were the cellular organelles that remained preserved during apoptosis of endocrine cells. Some of them appeared partially digested and in continuity with the colloidal materials (Fig. 17A-D). In the cytoplasm of FS cells, numerous dense spherical bodies regarded as lysosomes, mitochondria and secretory vesicles with colloid-like inclusion materials were also frequently detected. Some FS cells with phagocytosed endocrine cells not in contact with colloid were observed. But they were connected with other FS cells localized adjacent to colloid masses (Fig 18A-C).

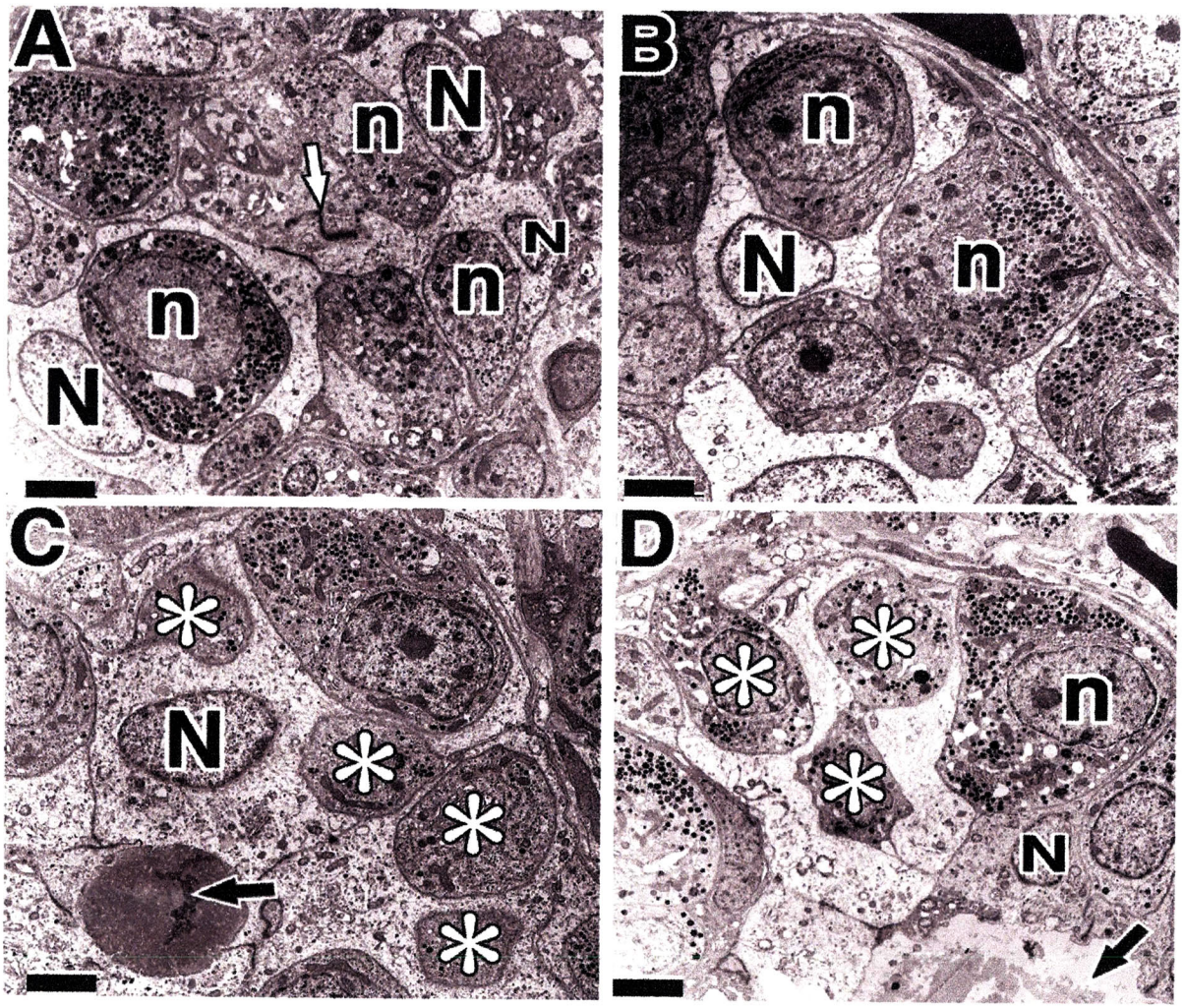

Fig. Electron micrographs of endocrine cells engulfed by FS cells and transformed into apoptotic bodies. (A, B) Morphologically intact apoptotic endocrine cells (n) are observed in the cytoplasm of FS cells (N). A prominent junctional complex (open arrowhead) in the lateral region represents an early stage of follicular development. (C, D) Show phagocytosed endocrine cells (n) manifesting typical features of apoptosis including; membrane blebbing, cytoplasmic shrinkage, nuclear condensation and formation of membrane-bound fragments [apoptotic bodies] (asterisks) in the cytoplasm of FS cells (N) surrounding colloid masses (arrows). Scale bar: 500nm

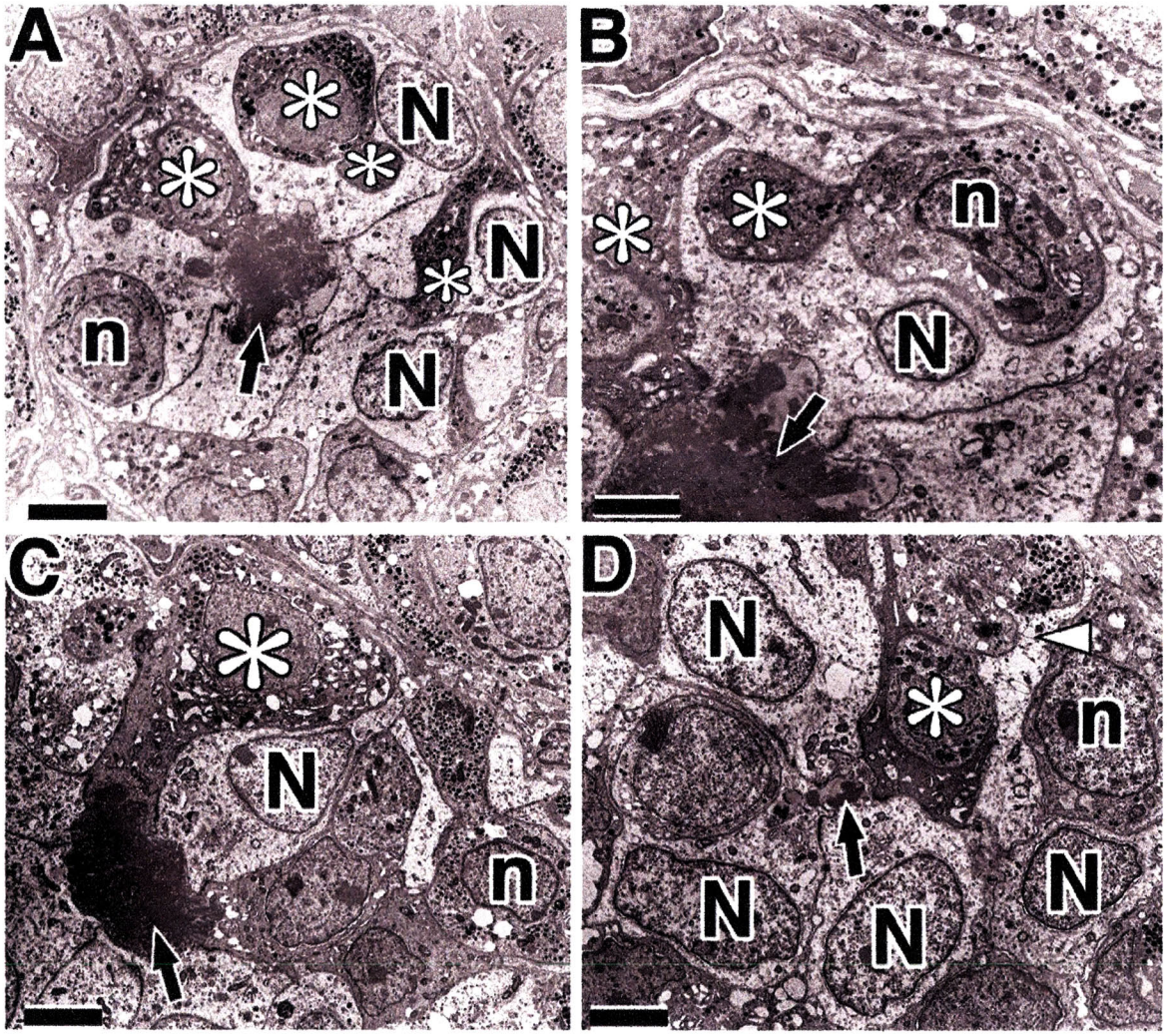

Fig. 17 Electron micrographs of partially digested apoptotic endocrine cells. (A-D) Show partially digested apoptotic bodies of endocrine cells (asterisks) can be observed in the cytoplasm of FS cells (**N**) and are in continuity with the colloidal materials (arrows). Open arrowheads is cytoplasm of FS cell. Scale bar: 500nm.

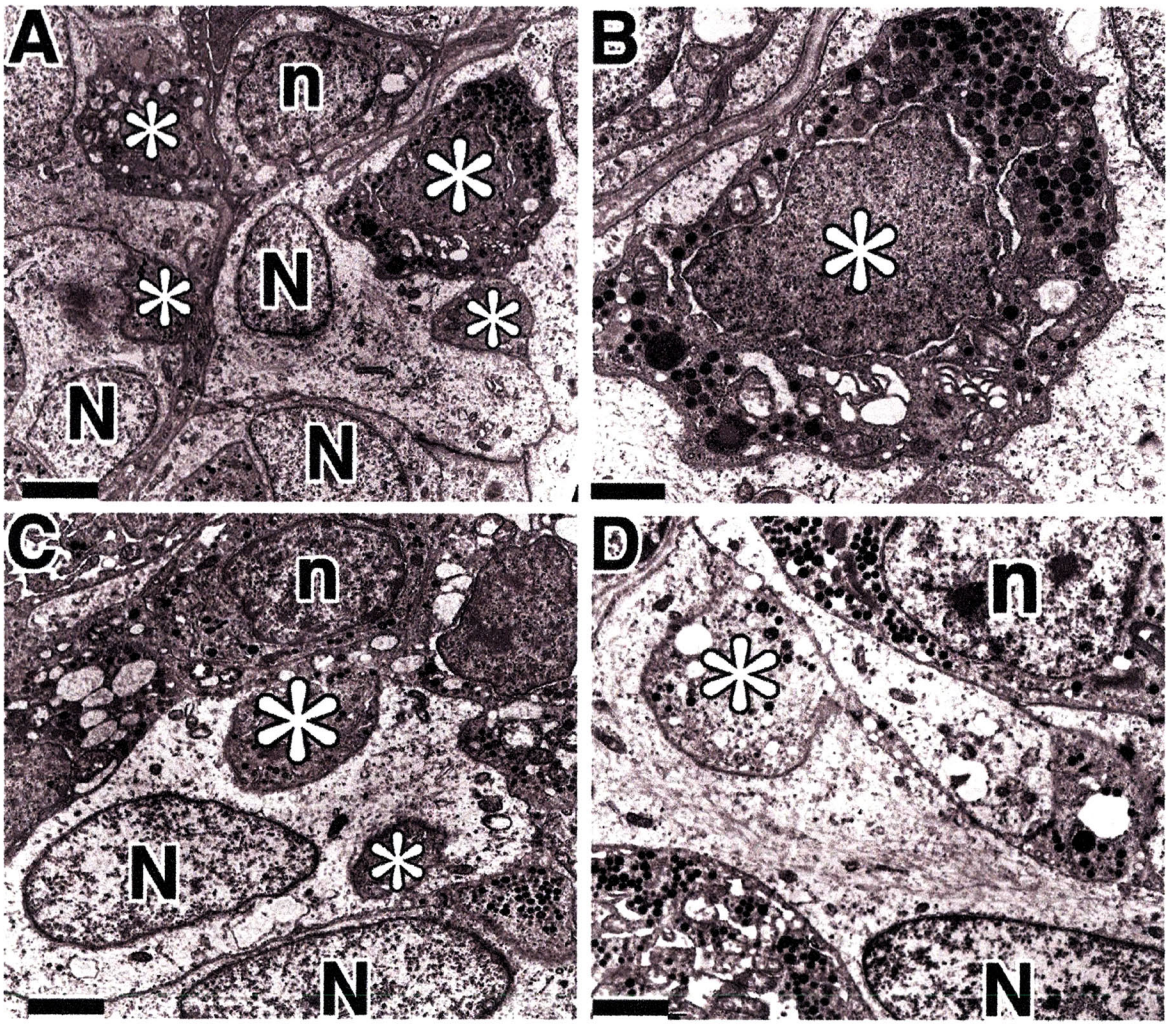

Fig. 18 Electron micrographs of apoptotic bodies-containing FS cells not localized adjacent to colloidal structures. (A-D) Show FS cells (**N**) with apoptotic bodies (asterisks) localized away from colloidal structures, but connected to other FS cells. (B) Higher magnification of (A) showing an FS cell containing an endocrine cell with typical features of apoptosis such as membrane blebbing, cytoplasmic shrinkage and nuclear condensation. Scale bar: A: 1.5μm; B: 250nm; C, D: 500nm.

5.4 DISCUSSION

Extracellular accumulations of colloid have been reported within the pituitary gland of several mammalian species including man, bat, cattle, dog, guinea pig and viscacha (Bassett, 1995; Kagayama, 1965; Fukuda, 1973; Anthony and Gustafson, 1984; Ciocca et al., 1984; Kameda, 1991; Kubo et al., 1992; Mohamed et al., 2000). In this study, we describe these colloids in the guinea fowl. The colloids were PAS positive and completely surrounded by FS cells. Similar morphological features were reported by Ogawa et al. (1996), Kagayama, (1965) and Fukuda, (1973) in other animal species, suggesting that large parts of these colloids are produced by FS cells. Immunohistochemical analysis to determine the biochemical composition of colloids confirmed that they contained clusterin protein which was localized in the cytoplasm of some FS cells and endocrine cells. Clusterin was not detected in all endocrine cells and FS cells, and this could be associated with the hormonal status of the animal and the function of individual cells at the time point when they were sampled. A normal cell responds to stress stimuli or damage 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.

The observation of clusterin in colloids is consistent with findings made by Ogawa et al. (1997), who purified and analyzed clusterin from colloids in the senescent porcine anterior pituitary and with Inoue et al. (2002), who observed clusterin protein localization in endocrine cells. Moreover, our findings on clusterin mRNA expression in the anterior pituitary of guinea fowl coincide well with published reports on clusterin mRNA expression in the pituitary gland of cows and sheep (Laslop et al., 1993;

Fleming et al., 1999). We also observed clusterin mRNA and protein in FS cells in the guinea fowl, and this has not been reported in other animal species.

In order to assess whether clusterin mRNA occurs within cells undergoing apoptosis within the anterior pituitary, we performed in situ hybridization for clusterin mRNA, followed by immunohistochemistry for ssDNA in the same section. Cells labeling for clusterin mRNA and ssDNA positive cells (apoptotic cells) were distinct, and therefore clusterin mRNA appears to be produced not by apoptotic cells but by neighboring, surviving cells. Our data support the results of previous investigations in vitro (French et al., 1994) and in vivo (Michel et al., 1997) showing that under low or less toxic stimuli, the clusterin gene is transcribed in surviving cells located near apoptotic ones, and hence raising the hypothesis of a cytoprotective role of clusterin within a tissue where apoptosis is occurring. However, under high levels of cytotoxic stress, uncleaved, non-glycosylated disulfide-linked isoforms of clusterin accumulate in the nucleus and contribute to cell death (Leskov et al., 2003; Trougakos and Gonos, 2004).

The detection of clusterin in endocrine cells, FS cells and in the colloids indicates that endocrine cells produce clusterin to exert cytoprotective effects before apoptosis. However, under high cytotoxic stress they undergo apoptosis. Apoptotic endocrine cells are phagocytosed by FS cells and digested by lysosomal enzymes. In FS cells, clusterin interacts and aggregates with molecules, proteins and peptides resulting from this digestion and subsequently become stored in the follicular lumen as colloid. In support of this idea, endocrine cells at different stages of apoptosis were observed to be phagocytosed by FS cells. The phagocytotic activity of these cells has also been clearly demonstrated in the pituitary gland of estrogen-deficient rats, where estrogen deficiency

is known to induce apoptosis of prolactin cells (Drewett et al., 1993). The observation of partially digested apoptotic endocrine cells being in continuity with the colloidal materials suggests further that these cells are transformed into colloids. Numerous dense spherical bodies identified as lysosomes, mitochondria and secretory vesicles with colloid-like inclusion materials were frequently detected in the cytoplasm of FS cells. These vesicles seem to represent one stage in the course of colloid formation and accumulation. FS cells located away from colloids probably dispose of their by-products via neighboring FS cells that are in contact with the colloidal accumulations.

Previous studies have provided experimental evidence that clusterin contains putative amphipathic alpha-helices that mediate hydrophobic interactions with numerous types of molecules (Jenne and Tschopp, 1989; Tsuruta et al., 1990) and may be involved in the clearance of cellular debris caused by cell injury or death (Bailey et al., 2002). Clusterin also has a heterogeneous charge attributable to the carbohydrate moieties that are sulfated and contain sialic acids. Most of the N-glycosylation sites are found within, or close to, the disulfide bonds and may form scaffolded regions in clusterin with negatively charged carbohydrates localized in this scaffold (Bailey et al., 2001). Consistent with this, the colloid in the anterior pituitary was positive for clusterin protein as well as for PAS reaction identifying carbohydrate. In general, our results demonstrate that clearance of apoptotic endocrine cells by phagocytotic activity of FS cells is the main feature in the anterior pituitary that leads to colloid accumulation.

CHAPTER VI

6.0 GENE EXPRESSION AND IMMUNOHISTOCHEMICAL LOCALIZATION OF MEGALIN IN THE ANTERIOR PITUITARY

6.1 INTRODUCTION

Megalin/LRP-2, a member of the low density lipoprotein (LDL) receptor family is shown to be abundantly expressed in the kidney, specifically in the cells of the proximal tubule and glomerular epithelium (Rodman et al. 1984; Farquhar et al. 1994) and to a lesser extent in a variety of epithelia including epididymis, type II pneumocytes, labyrinth epithelium of the ear, ciliary body of the eye, lacrimal gland, thyroid, parathyroid, ependymal and choroid plexus in brain (Kounnas et al. 1993, 1995; Zheng et al. 1994; Lundgren et al. 1997). Megalin is located in clathrin coated pits (Kerjaschki et al. 1984) where it serves as an endocytic receptor for a number structurally and functionally diverse proteins, polybasic drugs (Moestrup et al. 1995; Cui et al. 1996) and several polypeptide hormones found in the glomerular filtrate (Orlando et al. 1995). It plays a role in various biological processes including reabsorption of filtered proteins by cells of the proximal tubule (Moestrup et al. 1994, 1995; Orlando et al. 1995), endocytosis of proteins by yolk sac epithelia (Leung et al. 1989), clearance of proteases and protease inhibitor complexes from the alveolar space by type II pneumocytes (Poller et al. 1995; Stefansson et al. 1995) and in endocytosis and transport of clusterin and its complexes with the amyloid beta-peptide across vascular membrane, including blood-brain barrier and blood-cerebral spinal fluid barrier at the choroid plexus (Zlokovic et al. 1996).

Studies show that megalin binds clusterin with high affinity (Kd=14nM) and mediate its endocytosis and lysosomal degradation in cultured cells (Kounnas et al., 1995). In the pituitary gland clusterin has been detected in the colloids, FS cells and endocrine cells (Luziga et al. 2005; 2006). However, its expression and functional role in the pituitary gland has not been demonstrated. In this study we quantified by real-time PCR the relative expression level of megalin mRNA in pituitary and other endocrine tissues. Localization of megalin protein in pituitary was investigated by immunohistochemistry, immunoelectron microscopy and western blot analysis. The kidney was used as a reference because of its abundant expression of megalin mRNA and protein.

6.2 MATERIALS AND METHODS

6.2.1 Animals and tissue preparation

All experiments conformed to the law of 1973 concerning the protection and control of animal experimentation in Japan. Thirty six adult guinea fowls, aged 24 months were studied. They were anaesthetized with pentobarbital (0.8ml/kg iv) and brain, pituitary, lung, liver, spleen, pancreas, kidney, oviduct, ovary and testis were quickly excited and processed for quantitative real-time PCR. Thirty (30) milligrams of each tissue was treated with 300ul RNA*later* RNA stabilization reagent, incubated overnight at 4°C and then kept at -85°C until RNA extraction. Similar tissues were collected for comparison purpose from 12 months old Wistar rats (Institute of animal reproduction; Ibaraki, Japan). The rats were sacrificed under pentobarbital anaesthesia (60mg/kg). For western blot analysis Kidney, liver, pituitary, brain, ovary and testis from the guinea fowl were dissected, snap frozen in liquid nitrogen and stored at -85°C until required. Rat kidney and liver were also collected for used as positive and negative controls. For

immunohistochemical studies the dissected pituitary glands and kidneys were fixed in Bouin's solution for 2h at 4°C before processing to paraffin wax and sectioning.

6.2.2 Isolation of total RNA and synthesis of megalin first-strand cDNA

Total RNA was extracted from guinea fowl kidney using RNeasy Protect kit (Qiagen Sciences, GmbH, Germany) and the concentration was measured by absorption at 260nm. Poly(A)+ RNA was purified with oligo(dT)-cellulose using oligotex-dT30 (Super) mRNA purification kit (TaKaRa, Japan) and cDNA was synthesized with oligo (dT) primer using first-strand cDNA synthesis kit (Amersham Biosciences, Uk). Using set 2 of primers (Table 1) designed from the published rat megalin nucleotide sequences (Saito et al., 1994), a 661bp fragment of guinea fowl megalin was identified. The fragment was ligated into pGEM®-T Easy Vector System II (Promega, USA) and the sequence was confirmed by DNA sequencing using ABI 3100 DNA sequencer and analyzed by ABI software.

6.2.3 Real-time PCR using SYBR Green I detection system

Using primer express software (Applied Biosciences), specific primers (set 4, Table 1) were designed on guinea fowl megalin cDNA synthesized above and purchased from Fasmac Co. LTD, Japan. Preparation of poly(A)+ RNA from harvested tissues and synthesis of cDNA templates was done as describe above. A 165bp fragment was amplified in real-time using a GeneAmp 5700® Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Amplification was carried out in a total volume of 50µl containing 0.5X SYBR® Green I, PCR buffer [50mM KCl, 20mM Tris-HCl (pH8.3), 2.5mM $MgCl_2$, 0.2% glycerol and 0.2% dimethyl sulphoxide (DMSO)], 0.2µM each primer, 0.2mM dNTPs, 1.25U AmpliTaq Gold DNA polymerase (Applied

Biosystems) and 5µl of 1:5 diluted cDNA. The cycling conditions consisted of an UNG pre-incubation step at 50°C for 2 min, followed by a denaturation step at 95°C for 10 min and then 40 cycles of amplification that consisted of denaturation step for 15 s, 95°C, followed by annealing for 1 min, 60°C. A melting curve program was carried out from 60 to 95°C for 20 min for each individual sample amplified. The control cDNA was diluted from 1; 0.5; 0.25; 0.125 and 0.0625 and relative arbitrary quantities were defined. For comparison, real-time PCR was also performed on rat tissues. Procedure for tissue preparation was the same as described above except that specific primers (set 5, Table 4) were designed on the rat megalin (GenBank accession number L34049) and a 169bp fragment was amplified.

6.2.4 PCR data analysis

At the end of the PCR, the GeneAmp 5700® SDS software saved the results, allowing storage of the raw fluorescence data for further analysis. Some initial cycles were considered as a baseline or background in which no changes in fluorescence intensity occurred. The level above this at which increments in fluorescence became detectable was the threshold (Th). The software also determined the cycle number when a reaction reached the threshold. This value, termed the 'cycle threshold' (C_T), appeared during the exponential phase of the PCR and was inversely proportional to the initial number of template molecules in the sample. The software also computed a standard curve from the C_T values of the diluted standard and extrapolated absolute quantities for the unknown samples based on their C_T values. A baseline from 1 to 13 and a threshold of 0.07 were used for all determinations. Statistical analysis of the real-time PCR data was performed using a multifactorial analysis of variance (ANOVA) and Post-Hoc-Tests for

the paired differences between megalin mRNA expressed in the guinea fowl and rat tissues. The differences were expressed as significant at $P< 0.005$.

6.2.5 In situ hybridization

The method for in situ hybridization was performed using standard protocol (Sambrook and Russel 2001). For probes preparation, kidney tissue was used to isolate poly (A)+ RNA. To minimize cross hybridization to mRNAs encoding other members of LDL receptor family, hybridization probes were designed from 3'-untranslated (UTR) sequences as well as the coding region by selecting primers (Table 1) from the published rat megalin cDNA. The guinea fowl megalin cDNA sequences were amplified by PCR using the primer sets 1, 2 and 3 to generate 413bp, 661bp and 375bp fragments respectively. Each PCR product was ligated to pGEM-T Easy Vector (Promega, WI, USA) according to manufacturer's instructions. The cDNA inserts were checked by sequencing using ABI 3100 DNA sequencer with T7 and SP6 sequencing primers. The purified plasmids were linearized with Nco I and Nde I restriction enzymes and the RNA probes were labeled with digoxigenin-UTP by *in vitro* transcription with SP6 and T7 RNA polymerase using digoxigenin RNA labeling kit (Boehringer Mannheim). The procedure for in situ hybridization was essentially the same as described previously.

Table 4: Sequences of the designed primers sets (1, 2 , 3) for probe synthesis and real time PCR (4) using SYBR Green detection system

Primer sets	Product length	Forward primer (5'-3')	Reverse primer (5'-3')
1	413	Gaattctttatgctcatttgagggc	cagtcacgaaggtaggggacacctg
2	661	Gcttgttggactctttcactacagg	aggctatacgtcggaatcttcttta
3	375	Ggaattcgtagaatcaaacccgacg	ctggcaattaaatctaaggagtcat
4	165	Cgctagtggagacctggaag	cctcatctccacacaccaaa
5	169	Gactgtctggatgcgtctga	acaggtggatctcctcatcg

6.2.6 Immunohistochemical analysis

The method for immunohistochemistry was as described previously. However the primary antibodies used in this experiment were rabbit anti-megalin polyclonal antibody (diluted 1:200, Santa Cruz Biotechnology, Inc) and the rabbit anti S-100 protein polyclonal antibody (ready-to-use, Neo Markers, USA). The antibodies were applied to sections for 24 h in a dark, humid chamber at 4°C. Sections were washed (3X10 min) in PBS, followed by incubation for 30 min at RT with biotinylated goat anti-rabbit IgG (MP Biomedicals, Inc, Germany), both supplied ready-to-use.

To investigate the cellular pattern of megalin localization to that of clusterin within the anterior pituitary, double immunofluorescence was performed on pituitary sections to visualize the simultaneous localization of megalin and clusterin proteins. The initial steps in processing tissues remained the same as for streptavidin-peroxidase method. However, the sections were incubated for 24h at 4°C with a mixture of rabbit anti-megalin antibody (1:200) and mouse anti-clusterin antibody (1:200) diluted in PBS, pH 7.4. Sections were then washed (3X10min) in PBS followed by incubation with a mixture of Alexa Fluor® 488-conjugated donkey anti-rabbit IgG (FITC) and Alexa

Fluor® 546-conjugated goat anti-mouse IgG (TRITC) 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 Leica TCS SP Confocal microscope.

6.2.7 Immunoelectron microscopy

Pituitaries were rapidly excised and sliced into 1mm slabs in a few drops of pre-fixative solution (2% Paraformaldehyde, 2.5%Glutaraldehyde in 0.1M Sodium Cacodylate buffer, pH7.4 with 0.2mM $CaCl_2$). The tissues were then pre-fixed in the same pre-fixative solution for 90 minutes at 4°C with gentle shaking. After a rinse in 0.1M Cacodylate buffer, pH7.4, the tissues were post-fixed in 2%Osmium tetroxide for 90 min at 4°C followed by dehydration in graded alcohol series exchanged with acetone and embedding in epoxy resin (Quetol-651: NISSIN EM, Tokyo). Sections of 1μm thick were cut and stained with 1% toluidine blue and evaluated by light microscopy. Areas with follicular lumen were selected for fine trimming and thin sectioning. Ultra thin sections of approximately 70nm thick were cut and mounted on uncoated 300 mesh nickel grids (Ted Pella, Inc).

For immunoelectron analysis, sections were pretreated for antigen retrieval by soaking the grids in 0.01M sodium citrate buffer, pH 6.0 that had been microwaved to 90°C and kept on a hot plate for 10min and allowed to cool for 15min. After rinsing in distilled water, the grids were then placed in a blocking solution of 10% bovine serum albumin in 0.01M PBS; pH7.4 for 30min at room temperature followed by incubation with anti-goat megalin polyclonal antibody (diluted 1:200, Santa Cruz Biotechnology, Inc) overnight at 4°C. After washing in PBS, pH 7.4, the grids were incubated with rabbit

anti-goat IgG-Gold colloidal labeled-15nm (EY Laboratories, Inc) diluted 1:20 with PBS for 1h at room temperature, followed by two rinses in PBS. The grids were then stained with 2% uranyl acetate for 20 min and lead citrate for 7 min and examined at 80kV in transmission electron microscope (Hitachi H-7600 TEM Version 3.01).

6.2.8 Western blot analysis

Protein extraction was performed as previously described (Kerjaschki and Farquhar 1982) with minor modifications. In brief, tissues from the guinea fowl (kidney, liver, pituitary, brain, ovary and testis) and the rat (kidney and liver) were homogenized on ice in a buffer (1:4 w/v) containing 1%sodium deoxycholate (DOC)/50mM Tris-HCl, pH 8.6, 0.5mM phenylmethylsulfonyl fluoride, 0.5mM leupeptin, 150mMNaCl, 1.5mMMgCl_2, 10%(v/v) glycerol, and 1%(v/v) TritonX-100. After homogenization, samples were centrifuged at 120,000rpm for 20 min at 4°C to remove cell debris and nuclei and the supernatant was taken. Protein concentrations were measured by a Protein Assay kit (Bio-Rad Laboratories). Equal volume of 2X sodium dodecyl sulfate (SDS) buffer consisting of 2% SDS, 5% beta-mercaptoethanol, 10% glycerol in 50mM TRIS-HCl, pH 6.8 and 0.1% bromophenol blue was added to each sample and mixed well.

The samples were incubated at 100°C for 5 min followed by separation by electrophoresis in 4-12% density gradient polyacrylamide gel (Invitrogen, Inc) under standard conditions and then transferred onto polyvinlylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with 5% (w/v) BSA and 0.01% (v/v) Tween-20 in Tris-buffered saline (TBS, pH 7.6) for 1h at room temperature and was incubated with polyclonal goat anti-megalin antibody (1:500) in 2% (w/v) TBS-BSA overnight at 4°C. The membrane was then

washed (3X15) in TBS, after which a 1:5000 dilution of the donkey anti-goat IgG alkaline phosphatase linked secondary antibody (Santa Cruz Biotechnology, USA) was applied at room temperature for 1h. The membrane was then washed (3X15) in TBS and processed for development using an alkaline phosphatase substrate; bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Sigma), at room temperature.

6.3 RESULTS

6.3.1 Relative quantification of megalin mRNA measured by real-time PCR

Quantitative real-time PCR was used to measure the expression level of megalin mRNA in different tissues in the guinea fowl comparable to that of the rat in which megalin mRNA and protein are known to be abundantly expressed in the kidney. No significant deference was observed in the mean expression level of megalin mRNA in tissues from the rat and the guinea fowl. In both, highest levels were detected in the kidneys, followed by the pituitary and the lowest were seen in the liver. Result summary is presented in Table 5.

Table 5: Quantitative megalin mRNA expression measured by real-time PCR in various tissues collected from 24 months old guinea fowls and 12months old Wistar rats

S/N	Tissue	Megalin mRNA copies/mL	
		Guinea fowl	**Wistar Rat**
1	Brain	1.980±0.006	2.100±0.100
2	Pituitary	3.077±0.009	3.103±0.009
3	Lungs	2.480±0.012	2.470±0.015
4	Liver	1.067±0.015	1.043±0.009
5	Spleen	1.770±0.012	1.773±0.013
6	Pancreas	1.537±0.015	1.543±0.012
7	Kidney	4.267±0.015	4.277±0.015
8	Tests	1.720±0.006	1.700±0.006
9	Ovary	1.630±0.012	1.623±0.009
10	Oviduct	1.213±0.015	1.243±0.018

6.3.2 Expression of megalin mRNA in the anterior pituitary cells

The expression of megalin mRNA was detected in the cytoplasm of FS cells by the antisense digoxigenin-UTP labeled RNA probes. FS cells were recognized by their special tendency to surround colloids and extend cytoplasmic processes to surround adjacent endocrine cells. However, the distribution of hybridization signals did not appear to be associated with endocrine cells (Fig. 19A, B). In the kidneys, megalin mRNA was detected in the proximal tubule cells but not in the distal tubule cells (Fig. 19C). Hybridization signal was not observed in sections incubated with the labeled sense probe (Fig. 19D).

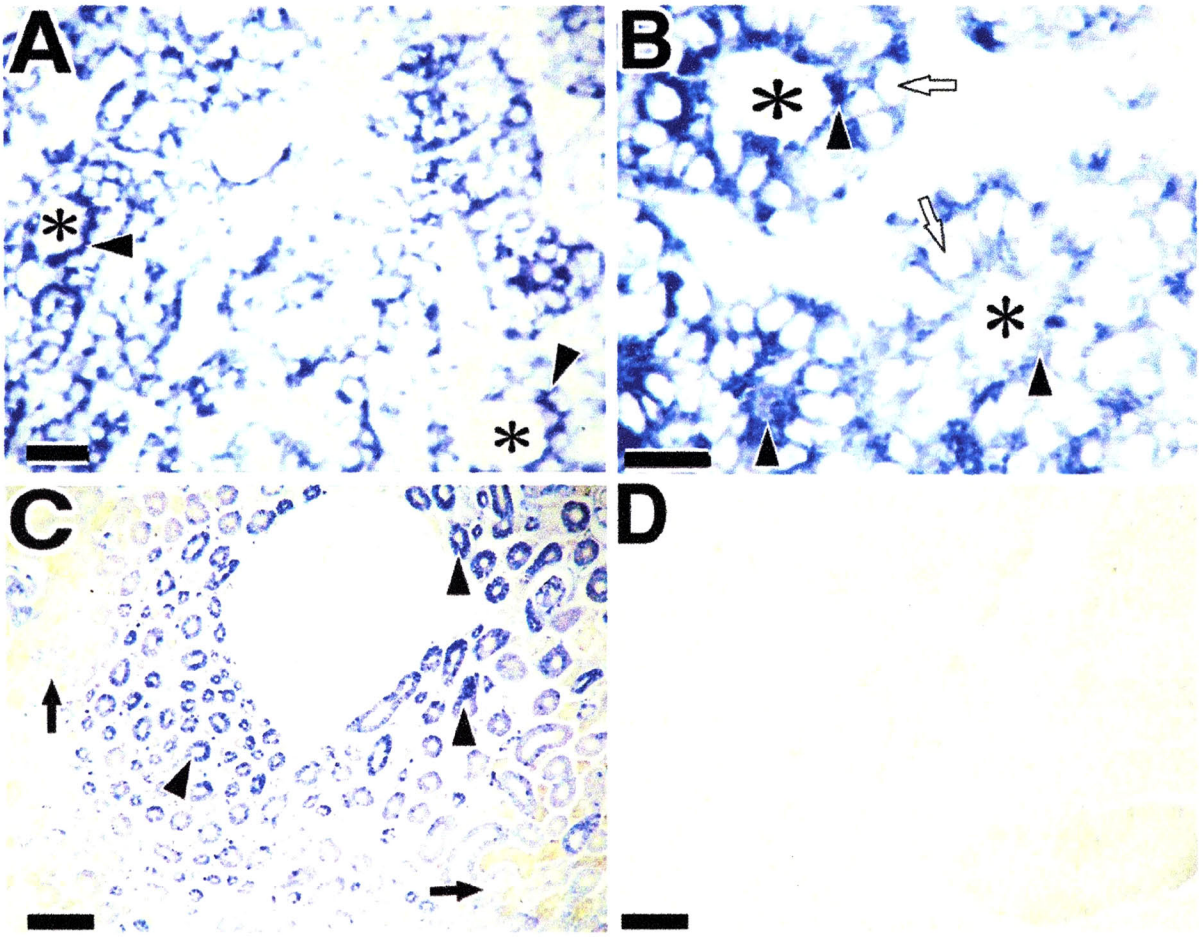

Fig. 19 Expression of megalin mRNA in FS cells. Megalin transcripts signal detected by in situ hybridization of digoxigenin-UTP labeled antisense probe is observed in FS cells (arrowhead) surrounding follicular lumen [asterisk] but does not appear to be associated with endocrine cells (Open arrows) [A, B]. In the kidney (C), hybridization signal is seen in epithelial cells of proximal tubules (arrowheads) but is absent in distal tubules (arrows) and in sections incubated with the labeled sense probe (D). Scale bar in A, C, D: 100μm; B: 20μm.

6.3.3 Simultaneous localization of megalin mRNA and S-100 protein in the same FS cells.

To confirm the expression of megalin mRNA in FS cells, after in situ hybridization, immunohistochemistry for S-100 protein was performed in order to localize FS cells. S-100 protein is contained specifically in FS cells in the mammalian pituitary; hence known as a marker protein of FS cells. This protein is therefore widely used in immunohistochemical studies to demonstrate FS cells (Nakajima et al. 1980). Overlap of brown colour of DAB reaction and blue colour of BCIP/NBT reaction enabled visualization of S-100 protein and megalin mRNA in the same FS cell in single section. However, not all cells that expressed S-100 protein also expressed megalin and hence some cells exclusively expressing megalin were observed showing blue colour only (Fig. 20A, B).

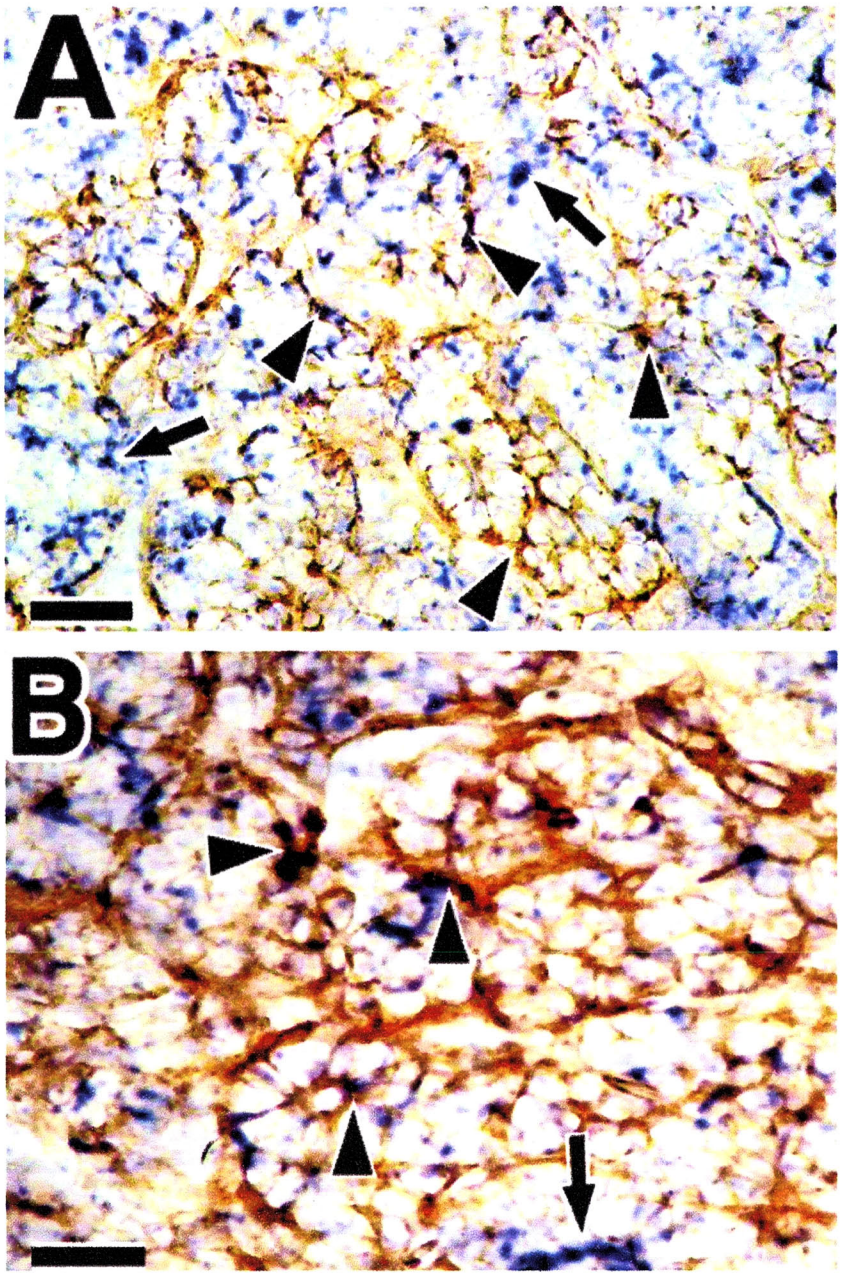

Fig. 20. Simultaneous expression of megalin mRNA and S-100 protein. Overlap of blue colour of BCIP/NBT reaction for megalin mRNA and brown colour of DAB enables visualization of megalin mRNA with S-100 protein (arrowheads) in the same FS cell (A, B). The simultaneous localization confirms the presence of megalin in FS cells. However, not all FS cells that expressed S-100 protein also expressed megalin and hence some cells exclusively expressing megalin appear distinctly blue (arrows). Scale bar in A: 100µm; B: 70µm.

6.3.4 Immunohistochemical localization of megalin in the anterior pituitary

Megalin protein detected by immunohistochemistry was observed in FS cells. Megalin positive FS cells were observed in the whole anterior pituitary surrounding almost all colloidal accumulations and extended long cytoplasmic processes to surround adjacent endocrine cells (Fig. 21A, B). The pattern of megalin protein localization in FS cells correlated well with the pattern of S-100 protein immunoreactivity when pituitary sections were labeled for binding of antiserum to S-100 protein (Fig. 21C). In the kidney, megalin protein was evident within the proximal tubule epithelial cells but was not observed in distal tubules (Fig. 21D).

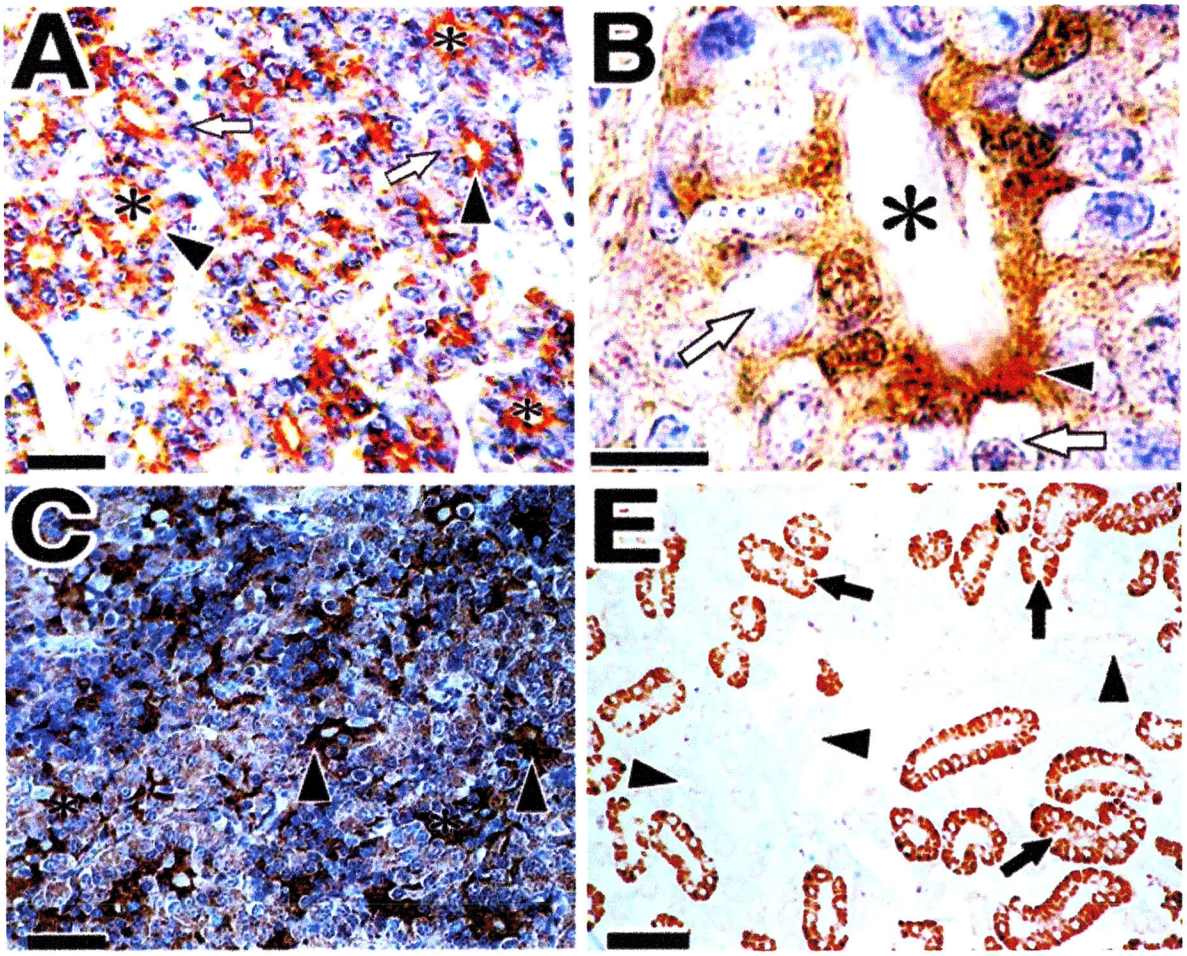

Fig. 21. Immunohistochemical localization of megalin protein in FS cells. Positive immunoreactivity for megalin (A) is evident in FS cells (arrowheads). FS cells are recognized by their special characteristics to surround colloid-containing follicles they produce (asterisks) and protrude long cytoplasmic processes to surround endocrine cells (open arrows). Higher magnification is shown (B). (C) Shows numerous colloids (asterisks) that are observed to be almost completely surrounded by S-100 positive FS cells (arrowheads). In the kidney (D), megalin positive immunoreactivity is seen in epithelial cells of proximal tubules (arrows) but is not observed in the distal tubules (arrowheads). Scale bar in A, C, D: 100μm; B: 20μm.

6.3.5 Simultaneous localization of megalin and clusterin in pituitary cells

To evaluate the cellular pattern of megalin localization to that of clusterin within anterior pituitary, double immunofluorescence labeling for megalin and clusterin was performed. Simultaneous localization of megalin and clusterin was observed in almost all FS cells. FS cell were recognized by their special tendency to surround colloid-containing follicles and extend long processes to surround adjacent endocrine cells. Many other isolated foci of colocalization were seen in other FS cells apart from those surrounding colloids (Fig. 22A-C).

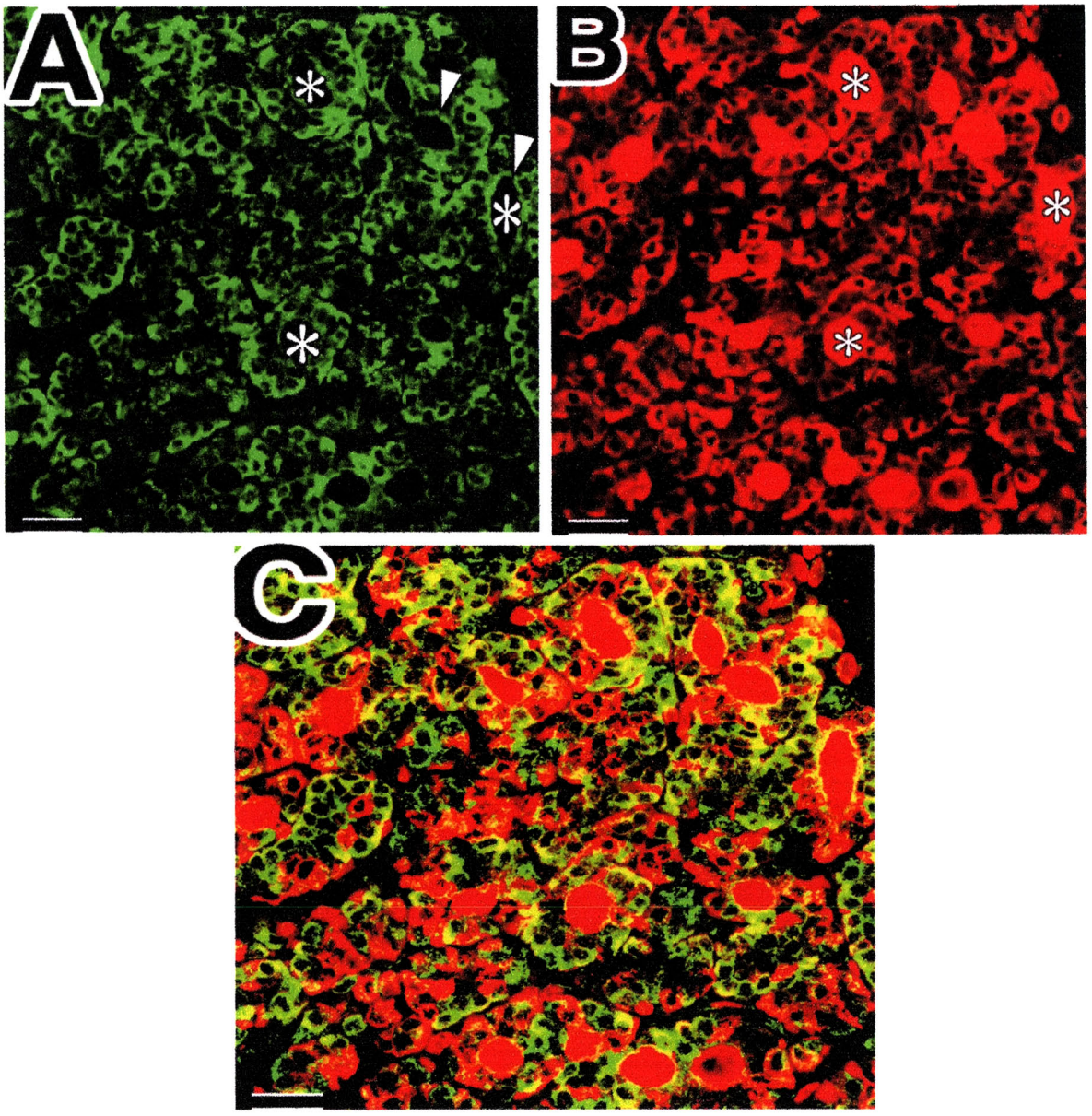

Fig. 22 Double immunofluorescence labeling for megalin and clusterin in the anterior pituitary gland. The photographs illustrate immunofluorescence labeling for (A) megalin (green, FITC), (B) clusterin (red, TRITC) and (C) merged image. Specific labeling for megalin (green) is seen in FS cells (open arrowhead) that are recognized by their tendency to surround colloids (asterisks) and extend long cytoplasmic processes to surround endocrine cells. Simultaneous localization of megalin and clusterin in FS cells is evident as yellow colour in the merged image. Apart from FS cells surrounding colloid-containing follicles, many other isolated FS cells are seen to be labeled for megalin that colocalizes with clusterin (yellow). Scale bar in A, C, D: 100μm

6.3.6 Immunoelectron microscopic findings

To define more precisely the localization of megalin protein in the anterior pituitary, we carried out immunogold labeling for megalin on ultrathin sections of pituitary gland. Specifically bound primary antibody was detected with secondary antibody conjugated to 15-nm colloidal gold particles. High density of gold particles was detected in the peripheral region of colloid-containing follicles and at a moderate level in the cytoplasm of FS cells (Fig. 23A, B). In the peripheral region of follicular lumen, megalin appeared to be associated with accumulating colloids. In FS cells, labeling was associated with secretory vesicles. However, immunogold labeling was not observed in the peripheral region of colloid-containing follicles and in the cytoplasm of FS cells in the control sections in which the anti-megalin antibody was replaced with rabbit IgG (Fig. 23C, D). Another population of cells that showed intense labeling for megalin was endocrine cells that were engulfed by FS cells in which labeling was observed to be associated with membrane of vacuoles. Most of the endocrine cells expressed megalin were apoptotic, being characterized by strong vaculolation, cellular shrinkage, nucleus condensation and membrane blebbing (Fig. 24A, B). However, immunogold labeling was not observed on membrane of vacuoles in the control sections (Fig. 24C, D).

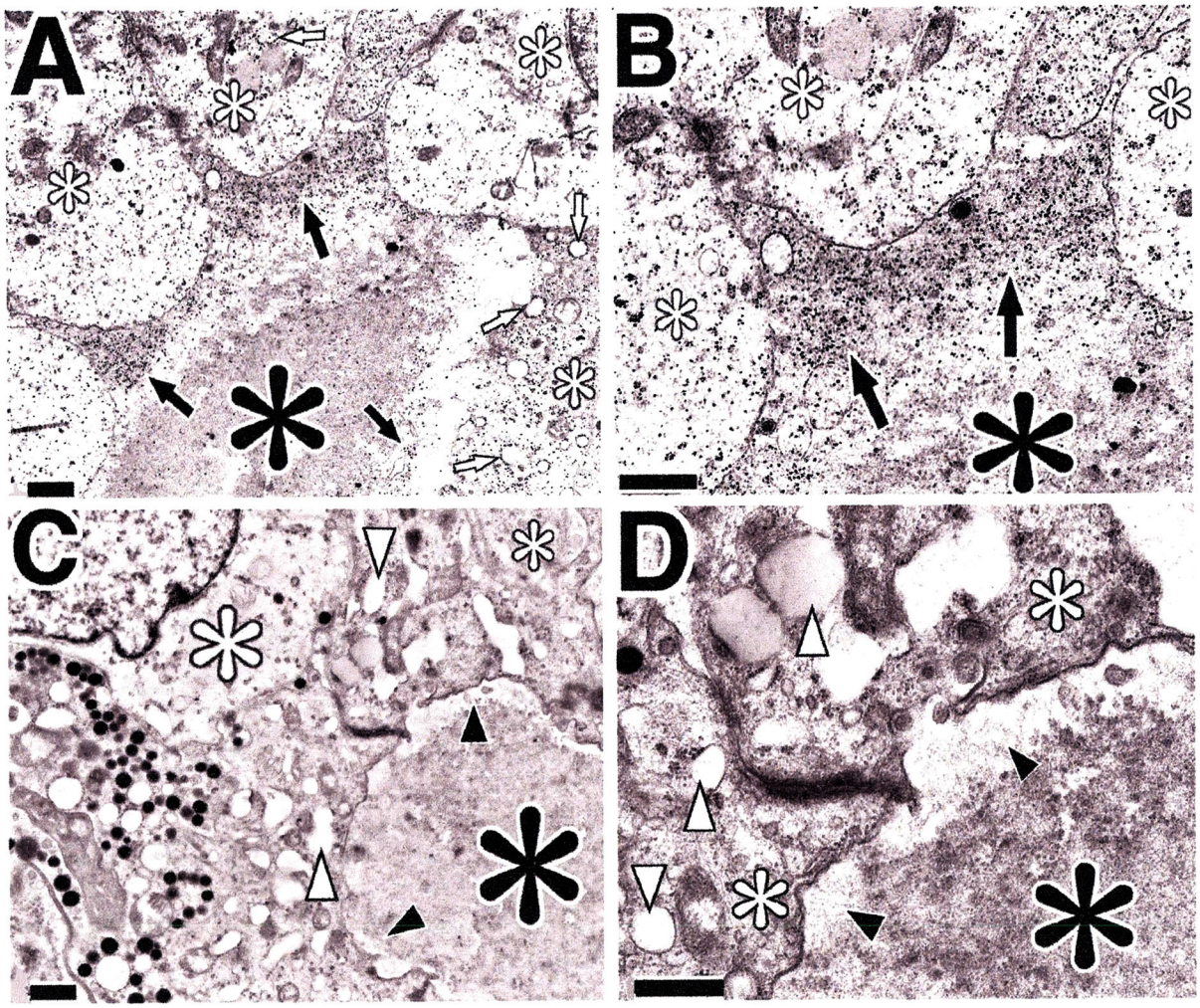

Fig. 23 Electron micrographs showing immunogold labeling for megalin in pituitary gland. (A) Illustrates presence of high density of gold particles (arrows) in peripheral region of colloid-containing follicle (black arrows) and at a moderate level in the cytoplasm of FS cells (open asterisks). In the peripheral region of the follicle, megalin appears to be associated with colloid coming for accumulation into the follicular lumen (black asterisks). In FS cells megalin is observed on membrane of vesicular structures (open arrows). Higher magnification of (A) is shown in (B). In the control sections, megalin immunoreactivity is not observed in FS cell (open asterisks), vesicular structures (open arrowhead) and in the peripheral region of follicular lumen (arrowhead) that contains colloid (black asterisk). Higher magnification of (C) is shown in (D). Scale bar in A, C: 2μm; B, D: 0.5μm.

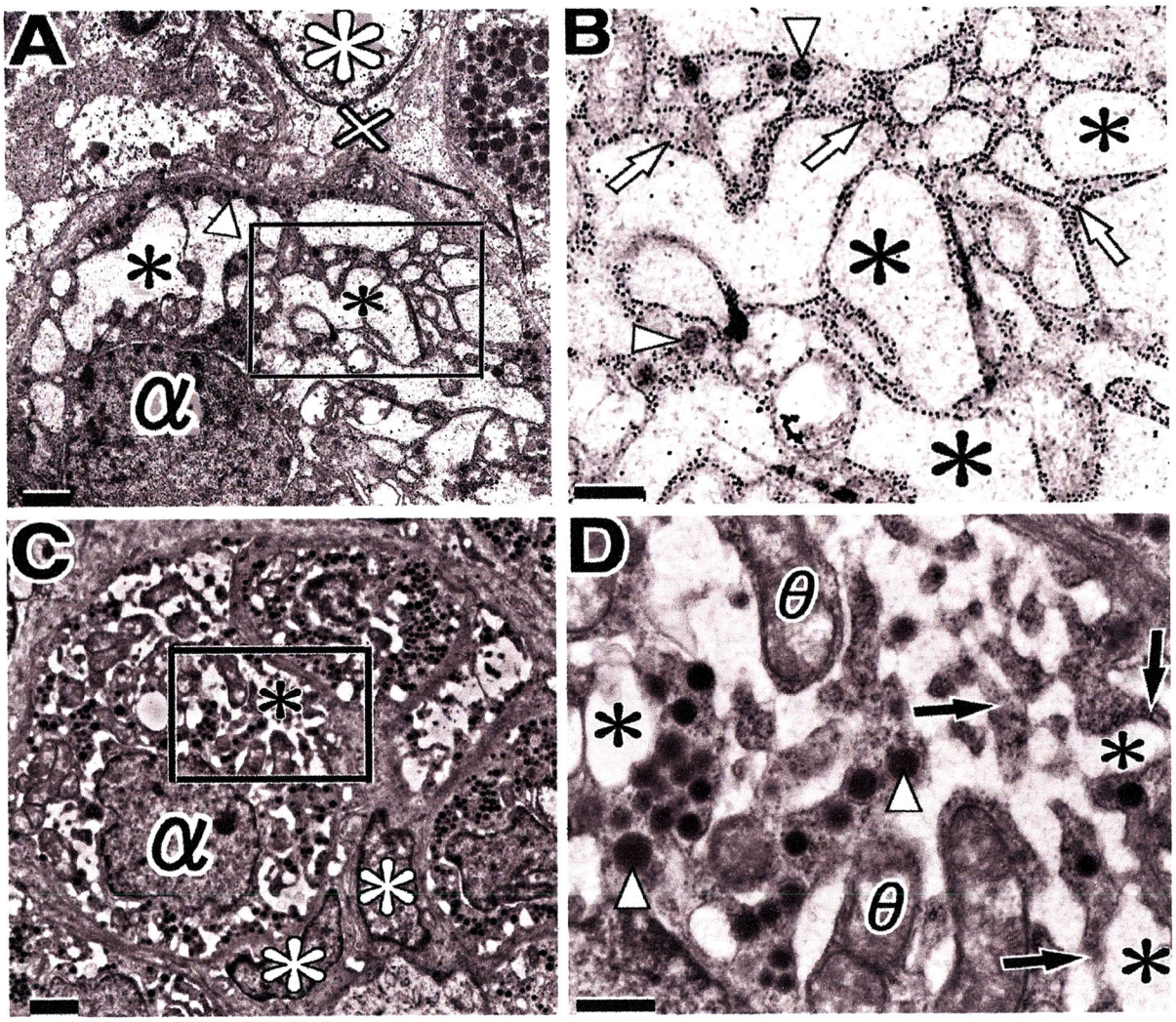

Fig. 24 Immunogold labeling for megalin in apoptotic endocrine cells. (A) Shows apoptotic endocrine cell (alpha) in the cytoplasm (cross) of FS cells (open asterisk). The endocrine cell manifests typical features of apoptosis including nuclear condensation, vacuolation (black asterisks) and contains a few granules (open arrowheads). A detail of immunogold labeling is shown at higher magnification (B) in which a high density of gold particles is seen on the membrane of vacuoles (open arrows). Vacuolation (black asterisks) and a few granules (open arrowheads) are evident. In the control section (C) of which the area of interest is shown at a higher magnification in (D); megalin labeling is virtually absent on membrane (black arrows) of vacuoles (black asterisks). Mitochondria (theta) and a few granules (open arrowhead) remain preserved during apoptosis of endocrine cells. Scale bar in A, C: 2μm; B, D: 0.5μm.

6.3.7 Detection of megalin protein in pituitary gland by western blot analysis

Western blot analysis of guinea fowl pituitary and kidney with the polyclonal antibody to megalin revealed a band with a molecular mass equal to that found in the positive control extract of the rat kidney. No megalin was detected in the extract from the liver, brain, ovary and testis as well as from the rat liver that was used as a negative control (Fig. 24).

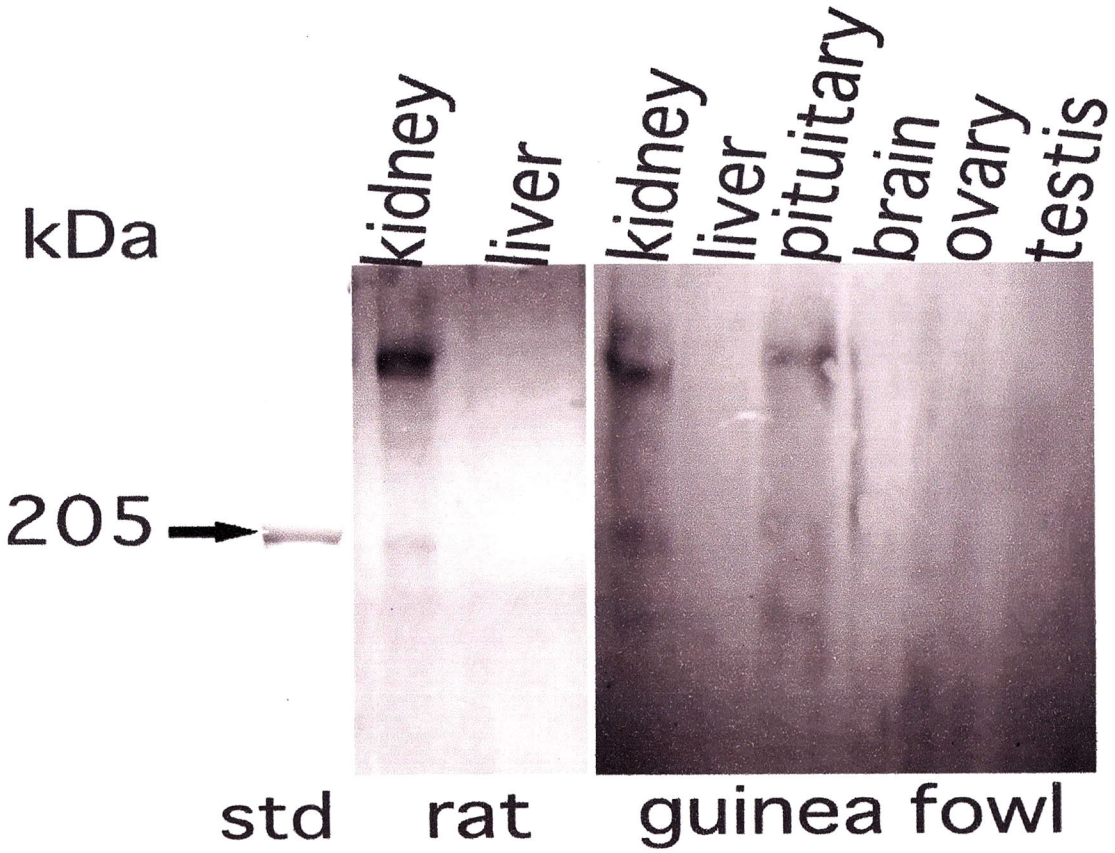

Fig. 25 Immunoblot analysis for the detection of megalin protein in pituitary gland. Protein homogenates from tissues of the rat and the guinea fowl were separated by electrophoresis in a 4-12% density gradient polyacrylamide gel and analyzed by immunobloting using an affinity purified goat polyclonal anti-megalin antibody raised against a peptide mapping near the C-terminus of megalin and donkey anti-goat IgG alkaline phosphatase linked secondary antibody (Santa Cruz Biotechnology, Inc). A band corresponding to megalin (600 kDa) is seen in the rat kidney (lane 1) used as positive control and matches well with that in the guinea fowl kidney (lane 1) and pituitary (lane 3). Megalin is absent in the negative control rat liver (lane 2) as well as in the guinea fowl liver (lane 2), brain (lane 4), ovary (lane 5) and testis (lane 6).

6.4 DISCUSSION

This study is the first demonstration of megalin protein in the pituitary gland with clear indications of its expression in FS cells. The pattern of megalin mRNA expression observed in various tissues in the guinea fowl was similar to that reported in other vertebrates (Kerjaschki and Farquhar 1983; Rodman et al. 1984; Farquhar et al. 1994), in which high levels were observed in the kidney. In the guinea fowl, megalin mRNA was particularly abundant in the kidney followed by the pituitary and to a lesser extent in the lung and almost none in the brain, spleen, testis, ovary, pancreas, oviduct and the liver. Similar pattern of expression was observed in the tissues from the rat that was used as a control animal. These results suggest that the biological function of megalin is well conserved in all species.

The in situ hybridization studies confirmed the findings of real time PCR by showing the expression of megalin mRNA in FS cells. To minimize cross hybridization to mRNAs encoding other members of LDL receptor family such as the LRP1, VLDL receptor (in chicken named LR8), LR11 also called SorLA and ApoER2 also known as LR7/8B, hybridization probes were prepared using primers that were designed from coding and the 3'-UTR region of the rat megalin, which is not shared by other members of the LDL receptor family (Gliemann 1998; Schneider et al. 1998; Nimpf and Schneider 2000). Results of immunohistochemistry supported in situ hybridization findings by clearly defining the localization of megalin protein in FS cells. Simultaneous expression of megalin mRNA and S-100 protein in the same FS cell confirmed further the ability of these cells to synthesize megalin. Specific localization of megalin in pituitary was also confirmed by western blot analysis. A similar band was observed in pituitary and kidney samples from the guinea fowl as was detected in the rat

kidney in which megalin is known to be abundantly expressed in the apical surface of proximal tubular epithelium (Kerjaschki and Farquhar 1983). These findings strongly suggest that megalin is truly produced in the pituitary gland.

The expression of megalin in FS cells that surround colloidal accumulations supports several hypotheses for the role of megalin in various epithelia facing transcellular fluids such as the thyroid fluid, yolk sac fluid, epididymal fluid, glomerular filtrate, cerebral spinal fluid and respiratory airways and indicates new and as yet undetermined roles for megalin in different tissues and organs. Similarly, our data do not contradict the known fact that megalin is mainly expressed in epithelial cells given that FS cells are the only epithelial element of the anterior pituitary (Couly and LeDouarin 1985; 1987). In addition, megalin is known to be a transmembrane protein with a large NH_2-terminal extracellular domain, a single transmembrane domain and a short cytoplasmic tail with NPXY motifs (Saito et al. 1994). The extracellular domain contains four cystein-rich complement-type repeats that constitute the ligand binding region (Davis et al. 1987). Detection of megalin in FS cells and in the peripheral areas of colloid-containing follicles indicates that megalin participates in the entire process of colloid formation and accumulation or reabsorption.

Affinity chromatography studies show that clusterin binds to megalin with high affinity (Kounnas et al. 1995) and its expression is enhanced in the face of tissue degeneration and apoptosis (Koch-Brandt et al. 1996). Similarly, megalin is shown to mediate not only clusterin endocytosis but also the clearance of clusterin and its complexes with the amyloid beta-peptide (Hammad et al. 1997) and the phagocytosis of clusterin complexes with membrane remnants and apoptotic bodies (Kounnas et al. 1995; Koch-

Brandt et al. 1996; Bartl et al. 2001). Consistent with our previous findings on clusterin in colloids, FS cells and endocrine cells (Luziga et al. 2005; 2006), it can be concluded that megalin drives ingestion of apoptotic endocrine cells through interaction with clusterin complexes in FS cells and hence providing a potential mechanism for a receptor mediated uptake of degenerating endocrine cells and secretion into follicular lumen as colloid.

CHAPTER VII

7.1 GENERAL DISCUSSION

This work describes the presence of extracellular colloidal accumulations in the pituitary pars distalis of the guinea fowl and discusses their origin, biochemical composition and biological significance. The observation of colloids in a 1-day-old bird indicates that their formation in this species of birds begins from prenatal life and that they form basic morphological structure which is related to certain biological functions in neonatal life. Previous studies on colloidal formation show that first appearance of colloid-containing follicles in the guinea pigs was recorded in 6month old animal (Kameda, 1991) and that they are rare and reduced in size in immature hamsters, hedgehogs and porcine (Spagnoli and Charipper, 1955; Ogawa et al. 1996; 1997). Results obtained in this study are essentially consistent with those observed previously (Ciocca et al. 1984; Kameda 1990; 1991; Kubo et al. 1992) in which the number of colloidal accumulations in various animal species increased with age.

Formation and development of colloid-containing follicles was observed to start within the cytoplasm of FS cells. Well-developed follicles were completely surrounded by FS cells indicating that major part of these colloids is produced by FS cells. However direct contact between colloid and granulated cells was also occasionally encountered. Such observation has also been reported in guinea pigs (Kameda 1990), suggesting that some granulated cells may be contributing to the formation of the colloid. From S-100 protein immunohistochemistry, positive labeling of S-100 protein was first observed in the marginal cells layer of Rathke's residual pouch in the pars distalis within the caudal lobe near the junction with pars nervosa (posterolateroal region). The other population

of positive cells was the FS cells located in the posterolateral region. In young birds, the first appearance of PAS positive colloids was also observed in this region, suggesting an existence of a relationship between colloids and FS cells. The observation in which S-100 protein antibody staining not only the FS cells located in the parenchymal tissue but also the supramarginal cells in the Rathke's pouch was also reported in other studies (Correr and Motta, 1985). This study therefore support the possibility that FS cells may have been developed from the rostral portion of the anterior wall of the Rathke's pouch, a structure derived from the oral cavity which is considered to be of ectodermal in origin.

It appears that the presence and number of colloids in senile birds may be a reflection of a change in the function of FS cells themselves with age. For this reason the importance of analyzing colloid formation become apparent. Colloids were observed to increase with age and the altered appearance of colloids in adults might be related to increased prolactin and gonadotrophin levels. It is worthy noting that many apoptotic cells probably lactotrophs and gonadotrophs, were observed to be clustered around colloid containing FS cells in adult birds. This phenomenon implies a relationship between FS cells and the endocrine cells associated with them. One possibility of how FS cells regulate endocrine cells is by secreting growth factors (Ferrara and Henzel, 1989) and altering their microenvironment such as their extracellular matrix. FS cells also produce many bioactive substances that influence endocrine cells such as 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 relationship of endocrine cells and FS cells with colloids in the anterior pituitary was confirmed further by electron microscopy and concluded that the phagocytic removal of apoptotic endocrine cells is the main feature that leads to accumulation of colloids. Apoptotic endocrine cells were observed to be phagocytosed by FS cells and the output of phagocytosis is accumulated in the colloids as residual bodies. The phagocytotic activity of FS cells has also been clearly demonstrated in the pituitary gland of estrogen-deficient rats by Drewett et al., (1993) who showed that FS cells phagocytosed apoptotic prolactin cells which appeared after the suspension of estrogen treatment or administration of dopamine agonists such as bromocriptine. In this study, the observation of partially digested apoptotic endocrine cells being in continuity with the colloidal materials suggests further that phagocytosed endocrine cells are transformed by FS cells into colloids. Numerous dense spherical bodies identified as lysosomes, mitochondria and secretory vesicles with colloid-like inclusion materials were frequently detected in the cytoplasm of FS cells. These vesicles seem to represent one stage in the course of colloid formation and accumulation. FS cells located away from colloids probably dispose-off their by-products via neighboring FS cells that are in contact with the colloidal accumulations.

Immunohistochemical analysis to determine the biochemical composition of colloids confirmed that they contained clusterin protein, which was also detected in the cytoplasm of some FS cells and endocrine cells. The observation of clusterin in colloids is consistent with the findings made by Ogawa et al. (1997), who purified and analyzed clusterin from colloids in the senescent porcine anterior pituitary and with Inoue et al. (2002), who observed clusterin protein localization in endocrine cells. Following the

identification of clusterin protein in colloids, FS cells and some endocrine cells, concerns regarding the specific cell type that synthesize clusterin were raised.

To investigate clusterin mRNA synthesizing cells and the expression pattern in the anterior pituitary, the full-length cDNA encoding guinea fowl clusterin was cloned and submitted to GenBank Sequence Database (accession number AY841278). Using this cDNA, RNA probes were made to be used for in situ hybridization analysis. Sequence analysis of the guinea fowl clusterin cDNA and comparison with the available avian clusterin cDNA sequences (chicken and quail) revealed a high degree of sequence homology and conservation of the predicted structural features of the proteins among the three species. Clusterin protein sequence around the known cleavage site in human clusterin (RIVR-cleavage-SLM) is different from that in the guinea fowl but identical to quail and chicken clusterin (RLSR-cleavage-ELH). However all three clusterin sequences in the birds appear to conform to cleavage site requirements for furin (R*XX*R) (Molloy et al., 1992). These findings suggest that there are evolutionary changes in clusterin among different species from the production of a single-chain protein in birds as reported in chicken (Mahon et al., 1999), to that of heterodimeric secreted form in mammals.

The pattern of clusterin mRNA expression observed in various tissues in the guinea fowl was similar to that reported in other vertebrates (Fleming et al., 1992; French et al., 1993; Wong et al., 1993; Mahon et al., 1999) in which high levels ware observed in the gonads. In the guinea fowl, clusterin mRNA was particularly abundant in the testis, ovary, brain and spleen and at lower levels in the liver, oviduct, pituitary, pancreas and lungs. These results suggest that the biological function of clusterin is well conserved in all species. The findings on clusterin mRNA expression in anterior pituitary of guinea

fowl coincide well with published reports on clusterin expression in the pituitary gland of bovine and sheep (Laslop et al., 1993; Fleming et al., 1999). The only exception to this is that clusterin mRNA and protein in FS cells in the guinea fowl was observed in this study. This phenomenon was not observed in other animal species. In this meaning, this study is the first confirmation of clusterin accumulation in the pituitary gland. Moreover, clusterin was not observed in all endocrine cells and FS cells at the same time, this could be associated with hormonal status of the animal and function of a cell at that time. A normal cell responds to stress stimuli or damage 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.

Previous studies on cells death show that clusterin appears before apoptosis in an apparent attempt by a cell to protect itself from cells death, although these cells for some reasons undergo apoptosis (Viard et al., 1999). Results obtained in this study are consistent with the hypothesis that endocrine cells produce clusterin before apoptosis to exert cytoprotective effect. Degenerating endocrine cells are phagocytosed by FS cells and digested by their lysosomal enzymes. In the FS cells, clusterin interacts and aggregates with molecules, proteins and peptides resulting from their digestion and subsequently become stored in the follicular lumen as colloid. This hypothesis is supported by the observation of clusterin in endocrine cells, FS cells and in the colloid. Involvement of clusterin in clearance of cellular debris is achieved by the presence of putative amphipathic α-helices that mediate hydrophobic interactions with numerous types of molecules (Jenne and Tschopp, 1989; Tsuruta et al., 1990).

In order to assess whether clusterin mRNA occurs within cells undergoing apoptosis within the anterior pituitary, in situ hybridization for clusterin mRNA was performed, followed by immunohistochemistry for ssDNA in the same section. Cells labeling for clusterin mRNA and ssDNA positive cells (apoptotic cells) were distinct, and therefore clusterin mRNA appears to be produced not by apoptotic cells but by neighboring, surviving cells. These data support the results of previous investigations in vitro (French et al., 1994) and in vivo (Michel et al., 1997) showing that under low or less toxic stimuli, the clusterin gene is transcribed in surviving cells located near apoptotic ones, and hence raising the hypothesis of a cytoprotective role of clusterin within a tissue where apoptosis is occurring. However, under high levels of cytotoxic stress, uncleaved, non-glycosylated disulfide-linked isoforms of clusterin accumulate in the nucleus and contribute to cell death (Leskov et al., 2003; Trougakos and Gonos, 2004).

Previous studies have provided experimental evidence that clusterin contains putative amphipathic □-helices that mediate hydrophobic interactions with numerous types of molecules (Jenne and Tschopp, 1989; Tsuruta et al., 1990) and may be involved in the clearance of cellular debris caused by cell injury or death (Bailey et al., 2002). Clusterin also has a heterogeneous charge attributable to the carbohydrate moieties that are sulfated and contain sialic acids. Most of the N-glycosylation sites are found within, or close to, the disulfide bonds and may form scaffolded regions in clusterin with negatively charged carbohydrates localized in this scaffold (Bailey et al., 2001). Consistent with this, the colloid in the anterior pituitary was positive for clusterin protein as well as for PAS reaction identifying carbohydrate.

In addition, clusterin is shown to bind to megalin with high affinity (Kounnas et al. 1995) and its expression is enhanced in the face of tissue degeneration and apoptosis (Koch-Brandt et al. 1996). In this study, the detection of megalin in FS cells associated with apoptotic endocrine cells and in the peripheral areas of colloid-containing follicles indicates that megalin participates in the entire process of colloid formation and accumulation or reabsorption. It is also possible that clusterin and megalin act together as a cellular waste management system within pituitary. Similarly, megalin is shown to mediate not only clusterin endocytosis but also the clearance of clusterin and its complexes with the amyloid beta-peptide (Hammad et al. 1997) and the phagocytosis of clusterin complexes with membrane remnants and apoptotic bodies (Kounnas et al. 1995; Koch-Brandt et al. 1996; Bartl et al. 2001). The findings of megalin in FS cells and colloid areas where clusterin is abundantly localized indicate that megalin drives ingestion of apoptotic endocrine cells through interaction with clusterin complexes in FS cells and hence providing a potential mechanism for a receptor mediated uptake of degenerating endocrine cells and secretion of colloid into the follicular lumen.

7.2 CONCLUSION

This study indicates that clearance of apoptotic endocrine cells by phagocytotic activity of FS cells is the main feature in the anterior pituitary that leads to colloid accumulation. Endocrine cells produce clusterin for cytoprotection before cell death. Clusterin in phagoytosed degenerating endocrine cells and that produced by FS cells may interact and aggregate with molecules, proteins and peptides resulting from lysosomal digestion in FS cells and subsequently become stored in the colloid as residual body. Analysis of clusterin fragments accumulated in the colloids would also be of great interest in understanding the function this protein in the pituitary gland. The observation of

megalin in FS cells and in peripheral region of colloid-containing follicles suggests that megalin is involved in endocytosis of apoptotic endocrine cells through interaction with clusterin and hence providing a potential mechanism for a receptor mediated uptake of apoptotic endocrine cells by FS cells and secretion of colloids.

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APPENDICES

APPENDIX I: Picture of adult *Numida meleagris galeata* Pallas

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Archosauria; Aves; Neognathae; Galliformes; Numididae; Numida; Numida meleagris galeata (helmeted guinea fowl)

APPENDIX II: Picture of young *Numida meleagris galeata* Pallas

The Helmeted Guinea fowl (*Numida meleagris*) is the best known of the guinea fowl bird family, Numididae, and the only member of the genus *Numida*. It breeds in Africa, mainly south of the Sahara, and has been widely introduced into the West Indies and Southern France. It breeds in warm, fairly dry and open habitats with scattered shrubs and trees such as savanna or farmland. It lays its large clutch of 20-30 eggs in a well-hidden lined scrape, and the females incubate the eggs for 26-28 days. These guinea fowls live as long as 12 years in the wild