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Review

Reversible Conversion among Subtypes of Salivary Gland Duct Cells as Identified by Production of a Variety of Bioactive Polypeptides

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Four major kallikreins (mK1, mK22, mK9, and mK13) were identified in the mouse submandibular gland (SMG). mK1, a true tissue kallikrein, was used as a protein marker to identify different types of SMG granular convoluted tubule (GCT) cells along with epidermal growth factor (EGF), nerve growth factor (NGF), and renin. Kallikrein mK1 was localized in a very small number (~5%) of GCT cells, which were scattered throughout the GCT, indicating that the majority of GCT cells are mK1-negative. Among mK1-positive cells, particularly strong signals were observed in a small number of narrow cells, recognized as slender granular cells (SG cells, Type IV), in the GCT. After postnatal development of the SMG, GCT cells are no longer uniform based on the bioactive substances (mK1, EGF, NGF, and renin) that they produce and secrete. GCT cells were classified into four subtypes, Types I–IV, and it became clear that these subtypes are complicatedly and reversibly converted by the endocrine hormones 5α-dihydrotestosterone (DHT) and triiodothyronine (T_3). Duct segments with similar morphology or hormone dependency were recognized in the sublingual and parotid glands. The presence of duct cells with such characteristics is therefore a common feature of the three major salivary glands of rodents.

Key words: 5α-dihydrotestosterone, epidermal growth factor, granular convoluted tubular cells, mK1, submandibular gland

I. Introduction

The fundamental functions of the salivary glands are to produce the primary saliva in the acinus, transport the saliva through the duct, and discharge the saliva to the oral cavity. The ducts, leading from the secretory end piece to the oral cavity, are composed of, in order, the intercalated duct, striated duct (SD), excretory duct, and main excretory duct. Major salivary glands have a lobular structure, and the main excretory duct is interlobular. In rodents, in the submandibular gland (SMG), one of the major salivary glands, the granular convoluted tubule (GCT) is located between the intercalated duct and the SD. This structure is unique to rodents and cannot be found in human salivary glands [1]. The GCT exhibits sexual dimorphism, and is changed markedly during postnatal development in animals, depending on the levels of endocrine hormones [8, 10]. In mice, the GCT is present in the SMG, and the sexual dimorphism is pronounced [2, 8]. Compared to the female gland, the male GCT is markedly hypertrophic, having a number of large secretory granules. It is known that the morphologies of male and female GCTs are dependent on sex hormones, thyroid hormones, and adrenocortical hormones [2, 8]. These hormones are dependent on the pituitary gland, and therefore hypophysectomized mice

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are effective experimental animals for examining the effect of individual hormones on the SMG. Using a hypophysectomized ICR mouse as an experimental model, we conducted a series of studies and examined the SMG by electron microscopy. The GCT cells were shown to undergo remarkable atrophy in hypophysectomized male mice: the secretory granules of GCT cells were decreased in size and number, and the basal infoldings of these cells were prominently developed. This observation indicates the conversion of GCT cells into immature GCT phenotypes. Androgens, thyroid hormones, and adrenal hormones were repeatedly administered to hypophysectomized male mice, individually or in combination, and the GCT phenotypes were precisely examined. The results indicated that the concomitant administration of androgen and thyroid hormones is essential for GCT cells to recover male phenotypes, and that it is possible to recover a structure close to the female phenotype mainly through thyroid hormones alone. Adrenal hormones act only supplementarily for the action of these hormones [18].

In humans, rats, and mice, salivary glands produce many kinds of bioactive polypeptides [1, 2, 7, 8]. Kallikrein is one such substance, and its existence has long been known. The kallikrein gene constitutes a large family, and major protein products of the family have been identified in the mouse SMG. One of them, mK1, the product of the tissue (renal) kallikrein gene *mKlk-1*, has been characterized as a true tissue kallikrein of mouse. Other typical bioactive polypeptides of the salivary glands are epidermal growth factor (EGF), nerve growth factor (NGF), and renin. Histochemical studies conducted with the production of mK1 and other bioactive polypeptides as indices are reviewed in this article. We will show that the morphologically indistinguishable GCT cells present in the duct segments can be divided into several subtypes due to differences in production of each bioactive polypeptide and in hormone sensitivity, and that they can be reversibly interconverted.

II. Salivary Gland Proteases and Their Physiological Functions

In 1968, Calissano and Angeletti [5] reported that they had purified two proteolytic enzymes, R-esterase and P-esterase, from the mouse SMG by measuring the hydrolysis activity of synthetic substrates. They also reported that these enzymes are induced by testosterone *in vivo*. Meanwhile, Boesman *et al.* [4] purified two enzymes, proteases A and D, from the mouse SMG. From their isoelectric points and behaviors towards cation and anion exchange resins, it was inferred that R-esterase but not P-esterase was the same enzyme as either protease A or protease D. Thus, protease A, protease D, and P-esterase were purified, and their physiological activities were investigated. Protease A of the SMG was shown to be the same enzyme as β-NGF endopeptidase, which performs restricted proteolysis of β-NGF, whereas protease D was found to be identical to the EGF binding protein, and the primary structures of these two proteins are similar [12]. Finally, a protease abundantly expressed in the female SMG, proteinase F, was found in ICR mice (CD-1 mice); its molecular weight was found to be 27 kDa, consisting of two subunits of 18 kDa and 10 kDa [14]. Ultimately, at this time, it was confirmed that there were four major proteases: protease A (β-NGF endopeptidase), protease D (EGF binding protein), P-esterase, and proteinase F, in the mouse SMG.

III. Identification of a Tissue Kallikrein, Its Relation to Protease, and Its Kinin-releasing Activity

Mouse tissue kallikreins family consists of many members, the DNA sequences of which were compared with the primary structures of various proteases reported in the past. Many of these proteases were found to be one of the tissue kallikreins, and a nomenclature was immediately proposed [3]. However, the existence of the protein for mouse tissue (renal) kallikrein (new name, mK1) was still unknown. Therefore, the primary structures and the kinin-releasing activities of the former four types of proteases were examined and compared. It was revealed that proteinase F was tissue (renal) kallikrein. It is also expressed in tissues other than the kidney and has the strongest kinin-releasing activity among the kallikrein family. These facts suggested that this enzyme is a tissue (glandular) kallikrein, or true tissue kallikrein, mK1 [16].

Before this information was uncovered, Mason *et al.* [30] and Evans *et al.* [6] reported that tissue kallikrein is comprised of a huge gene family consisting of 25–30 members, all located on chromosome 7. They also showed that all members have the same exon/intron gene structure, and that 14 genes encode proteins; pseudogenes that do not encode proteins are also present in kallikrein gene family. In addition, the primary structure of this family was compared with a series of serine proteases previously reported under various names, and a new nomenclature was approved for members of the tissue kallikrein gene family [3]. For example, mouse kallikrein genes and their proteins were designated as *mKlk-x* and mKx (where x is a number); the respective designations were *hKLKx* and hKx for humans, and *rKLKx* and rKx in rats. The chaos observed among related serine proteases was put in order, and many were revealed to be members of the tissue kallikrein gene family. At that point in time, the number of known tissue kallikrein genes was 26 in mice and 13 in rats, but for some reason only three in humans. However, later research on the human tissue kallikrein gene family revealed that there are 15 members in the human gene family [33]. Furthermore, many of these members were also reported to be upregulated by androgen, estrogen, progestin, or glucocorticoid [33].

At that point, the primary structures of proteases A and D (*i.e.*, β-NGF endopeptidase and EGF-binding protein) and P-esterase were analyzed and determined to be

Table 1. *Summary of hydrolysis of prorenin and kininogens (KG) by members of the tissue kallikrein family enzymes*

Tissue kallikrein	H-KG	L-KG	prorenin
	kinin formed (ng/μg/min)		prorenin processed (ng/μg/h)
mK1	1251 (100)	904 (100)	0 (0)
mK22	203 (17)	17.4 (1.9)	300 (18)
mK9	11.2 (0.9)	1.42 (0.1)	250 (15)
mK13	0.34 (0.02)	0.38 (0.03)	1660 (100)

The activity of kinin-generation was measured by using purified bovine H-KG and L-KG while prorenin processing activity, by using recombinant mouse prorenin. From reference 16.

identical to mK22, mK9, and mK13, respectively [16]. Together with the previously described tissue (glandular) kallikrein (*i.e.*, true tissue kallikrein) mK1, four major proteinases of the SMG had been identified. The kinin-releasing and prorenin-converting activities of these four types of kallikrein enzymes were examined using natural substrate proteins (bovine kininogen [KG] and mouse prorenin) and various synthetic substrates. The result showed that although mK1 had the strongest kinin-releasing activity, it became clear that its prorenin conversion activity was below the detection limit, and that mK13 showed an undetectable level of kinin-releasing activity but had the strongest prorenin-converting activity. The kinin-releasing and prorenin-converting activities of mK9 and mK22 were intermediate, falling between those of mK1 and mK13 (Table 1). Expression of the renin-angiotensin system and the kinin-kallikrein system by different members of the family was thought to regulate vasoconstriction/dilation [13, 16, 17]. Incidentally, mK13 also acts to process the precursor of interleukin-1β to produce active interleukin-1β (pleiotropic protein [31]).

Because mK1, a true tissue kallikrein, exists in the salivary gland and is localized in secretory granules of duct cells (see below), the localization of its substrate KG was also investigated [15]. There are four types of KGs: high molecular weight type (HMW), low molecular weight type (LMW), T-I, and T-II KGs, all of which are strongly expressed in the lung and liver. HMW-KG and T-I-KG were shown to be exclusively localized in the mast cells present in the SMG. No positive reaction was observed in the GCT cells, acinar cells, or SD cells. In the mast cells, KG was found to be localized in the secretory granules, but not in the nucleus or cytoplasm. These SMG mast cells are classified as the connective tissue type, and it is still unknown whether mucosal type mast cells, the other type of mast cell, express KG. The physiological meaning of the fact that KG is localized in the secretory granule of the mast cells in the SMG is also unclear.

IV. Localization of True Tissue Kallikrein mK1 and Polymorphism of Salivary Gland Duct Cells

As is well known, glandular cell-specific bioactive substances, such as various growth factors and hormone-like factors, are produced in and secreted from the duct cells [2, 8]. Usually, many of these bioactive substances are localized in secretory granules, and their contents in the glands also show marked sexual dimorphism (males > females). For the immunohistochemical study of mK1, a true tissue kallikrein, a specific antibody that recognizes only this enzyme was prepared [29]. Using this antibody, it was shown that mK1 is localized in only a very small number (~5%) of GCT cells scattered throughout the GCT in the adult male mouse SMG, and that the majority of GCT cells are mK1-negative (Fig. 1). Among the mK1-positive cells, particularly strong positive signals were found in a small number of narrow cells scattered throughout the mature male GCT; these cells were named slender granular cells (SG cells, Fig. 1) [25]. In adult females, there were no SG cells, and mK1-positive signals were detected in approximately 65% of typical GCT cells (Fig. 1). From the above results, it was revealed that mK1-positive and negative cells exhibit a unique mosaic distribution in the GCT of the SMG in both males and females, and that in males in particular, very few GCT cells expressing mK1 are present [25]. Furthermore, immunoelectron microscopy was performed using the colloidal gold method, confirming that mK1 is localized in the secretory granules of positive cells. The microstructure of SG cells found in the male GCT was examined. The Golgi apparatus and rough endoplasmic reticulum were extremely poorly developed in these cells, and mK1-positive small secretory granules were present at the apex; developed basal infoldings were also seen at the basal position. It was noted that SG cells are morphologically immature GCT cells [29]. Also, in the SMG, the amount of mK1 is reduced by androgen. Immunohistochemical analysis revealed that this reduction was not due to the decrease in mK1 production in individual cells, but was instead due to the decrease in the number of GCT cells expressing mK1 (Fig. 2).

In 24-week-old males, most of the mK1-positive cells were SG cells, suggesting that immature GCT cells exist in the male GCT (Fig. 2). In addition, the number of mK1-positive cells in males was increased by castration. The number of mK1-positive cells in both castrated males and normal females was decreased by androgen administration, and the number of mK1-negative cells increased in these

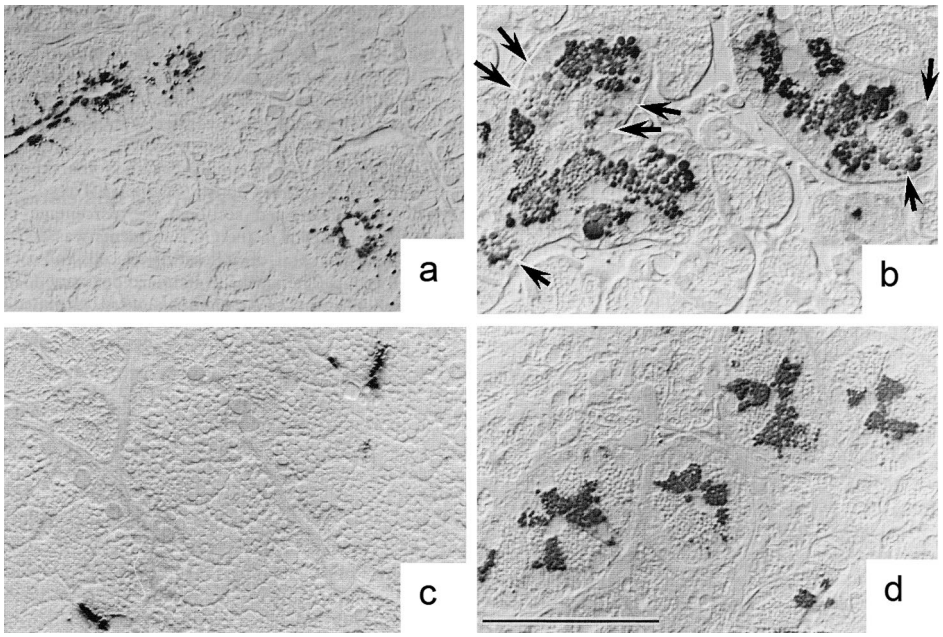

Fig. 1. Immunostaining of mK1 of the SMG in combination with Nomarski differential interference microscopy. The Epon/Araldite-embedded SMGs from developing and adult mice were sectioned at 2-μm and stained with pre-absorbed anti-mK1 antiserum. **a** and **b**, the SMGs from mice at 2 weeks and 4 weeks of age, respectively. **c** and **d**, the SMGs from an adult male and female, respectively. Immunonegative cells (arrows) appeared at 4 weeks of age (**b**). Bar = 50 μm. Modified from reference 26. Copyright©2002 (The Histochemical Society, Inc.) DOI: 10.1177/ 002215540205000202

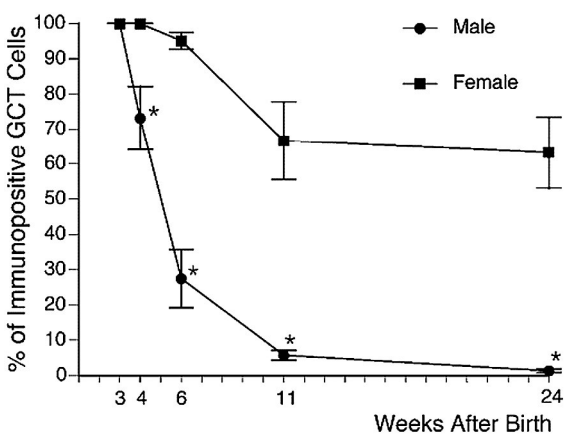

Fig. 2. Percentages of mK1-immunopositive GCT cells during postnatal development of male and female mice. Asterisks indicate statistically significant differences of $p < 0.001$ determined by Student's *t*-test between the means of the two sexes at the same age. From reference 26. Copyright©2002 (The Histochemical Society, Inc.) DOI: 10.1177/ 002215540205000202

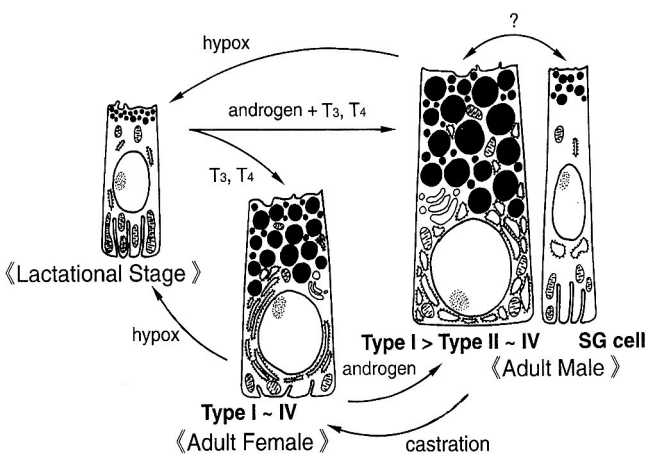

Fig. 3. Schematic demonstration of hormonal regulation of GCT cells. hypox, hypophysectomy; T_3, triiodothyronine; T_4, thyroxine. From reference 22.

mice. From the above results, it was revealed that mK1 is an enzyme specific for immature GCT cells, and that expression of mK1 decreases in many GCT cells in an androgen-dependent manner after reaching the sexual maturation stage [26]. However, this phenomenon was observed in ICR mice; when inbred mice were examined, no inhibitory effect of mK1 biosynthesis by hormone administration was observed, and all GCT cells of the mature male SMG were mK1-positive. The reason for the difference in expression regulation among different mouse strains is unknown at present.

V. Reversible Conversion of SMG Granular Duct Cells during Development and/or by Hormones

Here we summarize the previous reports of sexual dimorphism and hormonal regulation of the SMG GCT (Fig. 3) [22].

1) At 1 to 2 weeks after birth, in cells widely arranged

Table 2. *Immunocytochemical classification of GCT cells*

Cell type	Outcome of immunostaining			
	EGF	NGF	renin	mK1
I	+	+	+	−
II	+	+	+	+
III	+	+	−	−
IV	−	−	−	+

+, immuno-positive; −, immuno-negative. From reference 22.

along the SD, small secretory granules appear in the luminal area; these cells morphologically begin to differentiate into GCT cells (Fig. 3; lactational stage). At an early stage of differentiation, this segment is composed of a single cell group that produces and secretes only mK1. At 2 to 3 weeks of age, most of the cells begin to produce EGF and NGF in addition to mK1.

2) At 4 weeks after birth (sexual maturation initiation phase), renin-producing cells appear in a mosaic pattern, and accordingly, the number of mK1-negative cells increases. From this point on, the cells constituting the GCT are not homogeneous, but are instead divided into several subtypes. In the adult male GCT, in addition to SG cells expressing only mK1 (Fig. 3), GCT cells that all appear morphologically the same appear. These cells are classified into at least four subtypes, Types I–IV, depending on their production and secretion of bioactive substances. This was clarified by immunostaining methods using antibodies against the four types of bioactive substances (Table 2).

3) The female GCT phenotype is maintained mainly by thyroid hormones. In males, it has experimentally become apparent that thyroid hormones have a permissive effect on the actions of androgens, and that both androgens and thyroid hormones are essential for maintaining the male GCT phenotype. As shown in Figure 3, the phenotype conversion mediated by hormones is reversible, and all of the GCT cells become atrophied after hypophysectomy, returning to morphologically immature cells containing small secretory granules.

The effects of 5α-dihydrotestosterone (DHT; an androgen), dexamethasone (Dex), and triiodothyronine (T_3; a thyroid hormone) on expression of true tissue kallikrein (mK1) in the SMGs of hypophysectomized male mice were further analyzed. Although hypophysectomy caused the marked atrophy of GCT cells, the expression of mK1 was maintained, and almost all GCT cells were mK1-positive. When examined using colloidal gold immunoelectron microscopy, small secretory granules strongly positive for mK1 were observed to densely accumulate at the apical area in the atrophied GCT cells. The Golgi apparatus was poorly developed, the rough endoplasmic reticulum was sparse and scattered, and well-developed basal infoldings were observed at the basal area. These morphological features align with the morphological characteristics of immature GCT cells that appear during postnatal development. Administration of DHT, Dex, or T_3 hormones to hypophysectomized male mice improved the phenotype of the GCT cells. DHT and T_3 alone were thought to decrease the expression level of mK1 throughout the entire salivary gland by increasing the number of mK1-negative cells; Dex alone had no such effect. In addition, combined administration of DHT and T_3, or all three hormones, returned the phenotype of GCT cells to that of normal male GCT cells: many large secretory granules and a well-developed Golgi apparatus and rough endoplasmic reticulum appeared, whereas the amount of basal infoldings decreased. Only a few GCT cells were mK1-positive. The mosaic-like expression of mK1 in these GCT cells closely resembled the expression pattern in normal males. These results revealed that the sexual dimorphism of GCT cells and mK1 expression was not induced by administration of either DHT or T_3 alone, but was achieved by co-administration of the two hormones. In this case, T_3 had a permissive effect on the action of DHT, resulting in a phenotype unique to male GCT cells. mK1 was downregulated by the hormones, and its level was markedly decreased in many GCT cells. Thus, the number of mK1-negative cells was thought to be increased [27].

VI. Production, Localization, and Hormone Dependence of mK1, EGF, NGF, and Renin in the Sublingual Gland and Parotid Gland

In the mouse sublingual gland (SLG), EGF and renin are produced and secreted [9, 11, 32]. An examination by transmission electron microscopy revealed a duct segment composed of cells containing different sizes of secretory granules in the SLG. We examined and reported the effects of hormones on the cells present in this duct segment, which is similar to the SMG GCT [20].

In the SLG of adult male mice, many cells similar to those present in the GCT were scattered in the SD. In this segment, both mK1-positive cells and mK1-negative cells were observed. It was confirmed by immunoelectron microscopy that in mK1-positive cells, mK1 is localized in secretory granules. Light microscopic observation revealed that GCT-like duct cells in male mice atrophied after hypophysectomy, and that large secretory granules disappeared from these cells.

Administration of hormones (DHT, T_3, or Dex) restored the phenotype of the GCT-like duct cells, and large secretory granules reappeared. These results suggested that GCT-like duct cells of the mouse SLG are also regulated by hormones, and that androgen and thyroid hormones are necessary for phenotype expression and mK1 synthesis in a mechanism similar to that in the SMG GCT [21].

In the parotid gland of the mouse, there is neither a duct segment corresponding to the SMG GCT that contains large secretory granules, nor a duct segment in which GCT-

like cells are interspersed such as in the SLG. The localization of mK1 was studied using the enzyme antibody method [23]. In adult mice, accumulation of small secretory granules positive for mK1 was observed on the luminal side of the SD in both males and females. The size and number of secretory granules in males were somewhat superior to those in females, and a slight gender difference was recognized. Also, in castrated males, the size and number of secretory granules decreased, and in hypophysectomized males, secretory granules disappeared [23]. Furthermore, with combined administration of androgen and thyroid hormones, duct cells containing large secretory granules and similar to SMG GCT cells were induced in adult males, although in low numbers [19].

Hormones were administered to male mice, and their effects on the localization of mK1, EGF, NGF, and renin in the parotid gland were analyzed using the fluorescent antibody method. In the parotid gland of normal untreated animals, cells in the duct segment containing secretory granules showed positive reactions for mK1 and NGF. GCT-like duct cells, which were induced by co-administration of T_3 and DHT in normal males and closely resemble submandibular GCT cells, expressed mK1, EGF, and NGF. However, they were found to be negative for renin [19]. This result suggested that the phenotype of GCT-like duct cells induced in the parotid gland is equivalent to that of 3- to 4-week-old submandibular GCT cells [24]. These results show that the parotid duct also contains a duct segment that is constructed of hormone-dependent secretory cells, and emphasize that the granular duct is a common feature of the three rodent salivary glands.

At the 11th International Symposium on Exocrine Secretion, "Exocrine Secretion—Mechanism and Disease", in Tokushima, Japan in 2009, we reported on the pharmacokinetics of GCT-like granular duct cells induced in the parotid duct caused by administration of hormones. The salivary glands are innervated by both sympathetic and parasympathetic nervous systems, and their centers are located in thoracic spinal cord and superior/inferior salivary nucleus, respectively. The effect of methoxamine, an autonomic nerve agonist (sympathetic α-agonist), on the secretory response of granular duct cells was histologically examined using the three salivary glands of the male mouse that had been administered excess amounts of hormones. The SMG GCT cells promptly responded to this drug, and images showing the release of contents of the secretory granule were observed. The release of contents of the secretory granule also promptly occurred after drug administration in GCT-like duct cells induced in the parotid gland of the same individual. However, the secretory response of the GCT-like cells of the SLG could not be detected histologically, even at 1 hour after methoxamine administration. This result was quite interesting given that secretion by the SMG, parotid gland, and SLG is regulated by innervation of the same sympathetic nerve from the thoracic spinal cord (*via* superior cervical ganglion), and that the granular components of the granular duct cells of both glands are also almost the same [28].

VII. Conclusion

mK1, a kallikrein in the salivary gland, is a true tissue kallikrein, and the number of cells expressing it was decreased by androgen administration in ICR mice. This behavior is different from that of cells expressing EGF, NGF, and renin; that is, the number of cells expressing these bioactive polypeptides is increased by androgens. The sexual dimorphism of mK1 expression is based on two hormones: DHT and T_3. There are at least four kinds of cells, Types I–IV, in the SMG GCT if cells are classified according to their expression of mK1, EGF, NGF, and renin as indicators. Reversible conversion between the four types is thought to be controlled in a complicated manner by DHT and T_3. Hormone-dependent secretory cells also exist in the parotid duct. Furthermore, GCT-like duct cells are also scattered in the SD of the SLG, and bioactive polypeptides are produced. Therefore, it is concluded that the granular duct is a common feature of the three rodent salivary glands.

VIII. Abbreviations

Dex, dexamethasone; DHT, 5α-dihydrotestosterone; EGF, epidermal growth factor; GCT, granular convoluted tubule; HMW, high molecular weight; KG, kininogen; LMW, low molecular weight; NGF, nerve growth factor; SD, striated duct; SG cells, slender granular cells; SLG, sublingual gland; SMG, submandibular gland; T_3, triiodothyronine.

IX. Conflicts of Interest

There are no conflicts of interest to declare.

X. Acknowledgments

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XI. References

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