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ORIGINAL ARTICLE

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Organogenesis of the orbital glands in the lizard Podarcis s. sicula: a histological, histochemical and ultrastructural study

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Abstract The orbital glands of the lizard *Podarcis s. sicula* are represented by the anterior and posterior lacrimal glands and the Harderian gland. The anlage of the Harderian gland appears on about the 22nd day of development in the form of a short tubule projecting from the conjunctival epithelium. This event is coincident with the appearance of the nictitating membrane. At this stage the mesenchymal cells surrounding the glandular blastema proliferate at a high rate and form a definite sac, later occupied by both the Harderian gland and the anterior lacrimal glands. At the 26th day of development, the glandular blastema forms acini at its distal end. The prospective glandular cells are not yet differentiated histologically. At the 36th day of development, differentiated serous glandular cells become visible. At the 41st day of development, the acini fill up the preformed mesenchymal sac. Only at this stage does the most medial part of the gland differentiate into mucous-secreting anterior lacrimal gland. At the same time, a small primordium of the posterior lacrimal gland can be seen in the posterior commissure of the eye. The appearance of junctional complexes between epithelial cells and mesenchymal cells in the early developmental stages supports the role of the mesenchyme in the differentiation of the glandular cells. Since the glandular anlage differentiates laterally into Harderian gland and medially into anterior lacrimal gland, spatial and temporal differences seem to exist in the inductive process. Furthermore, a concentration gradient of the inductive substance(s) may be envisaged, since an intermediate zone is present between the Harderian gland and the anterior lacrimal gland, consisting of mixed glandular cells containing both mucous and serous secretory granules.

Key words Harderian gland · Lizard · Organogenesis · *Podarcis s. sicula*

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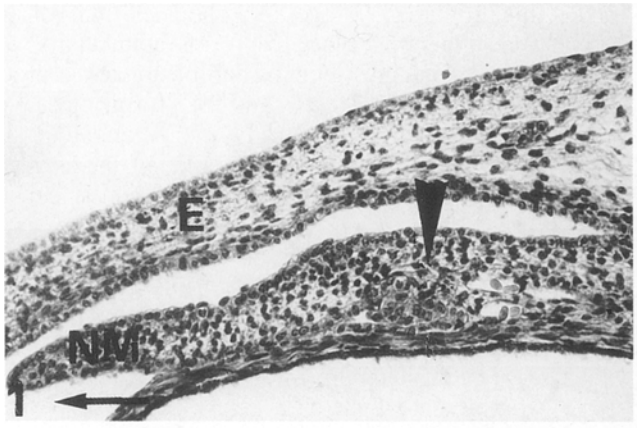
Introduction

Lacrimal glands (LG) and the Harderian gland (HG) are the largest of the orbital glands. The HG is present in those tetrapods that have a nictitating membrane, including amphibians, reptiles, birds and mammals, but not the primates. In recent years, these glands have been extensively studied with a variety of morphological, biochemical and physiological techniques in order to clarify their function. However, one of the less examined aspects is their embryological development. Old theories persist, according to which they have a common origin from a single LG precursor (see Payne 1994 for review). The purpose of this work is to describe the embryological development of the orbital glands of the lizard *Podarcis s. sicula* and to study, ultrastructurally, the differentiation of the glandular cells during pre- and postnatal life.

Materials and methods

Sexually mature males (n=50) and females (n=50) of the lizard, *Podarcis s. sicula*, were collected in the surroundings of Naples during the 1990, 1991 and 1992 breeding seasons. The animals were kept in a terrarium and fed with worms ad libitum. The eggs were taken as soon as they were laid, dated and placed in a container filled with moist soil. Humidity (60%) and temperature (26° C) were kept constant. On days 6, 12, 21, 22, 23, 24, 26, 27, 28, 32, 34, 36, 41 of incubation, the eggs were opened and the embryos collected. Newborn lizards were also sacrified at hatching (day 43) and 8 days later. Seriation of developmental stages was made according to the standard tables of Dufaure and Hubert (1961).

For histological and histochemical studies, heads were fixed in Bouin's fluid and 7-μm serial paraffin sections were stained with the haematoxylin-eosin, Mallory's trichrome, PAS and Alcian Blue/PAS (AB/PAS) techniques.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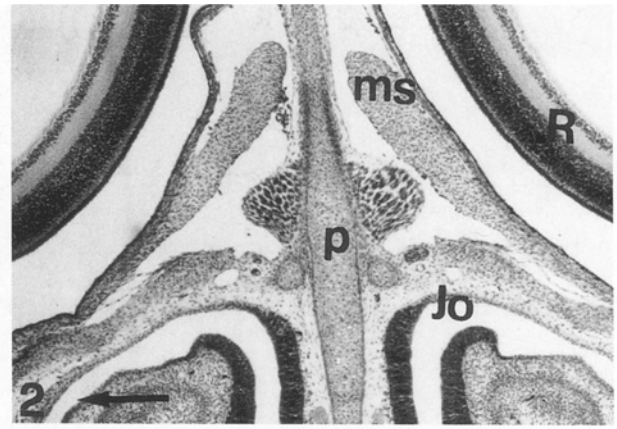
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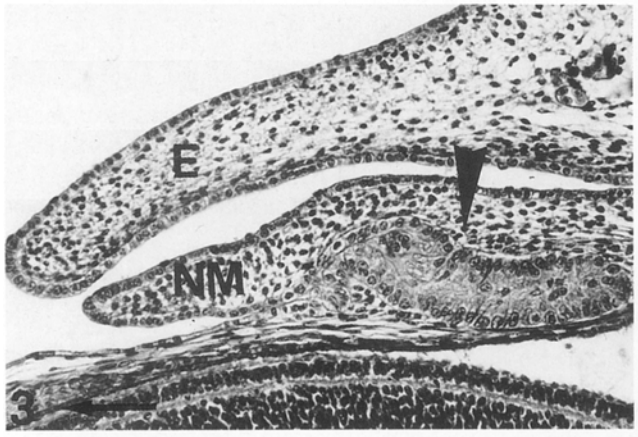
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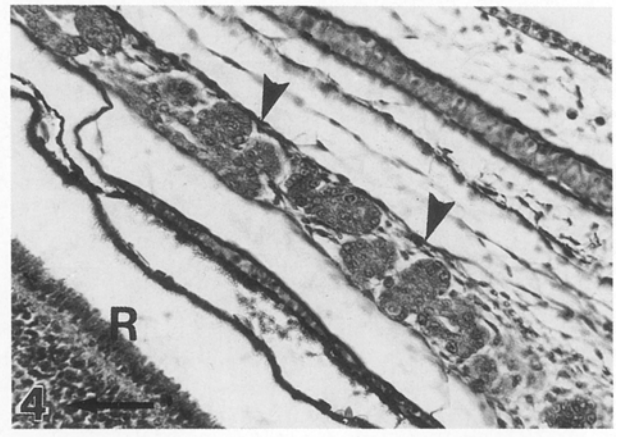
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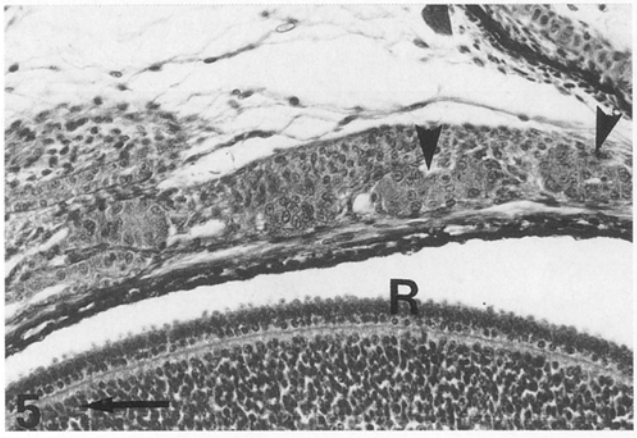
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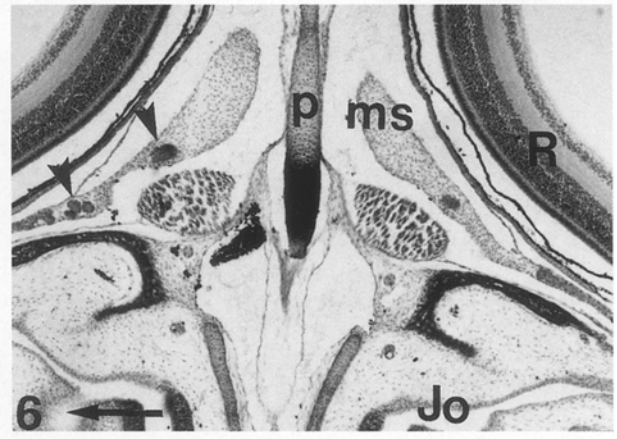
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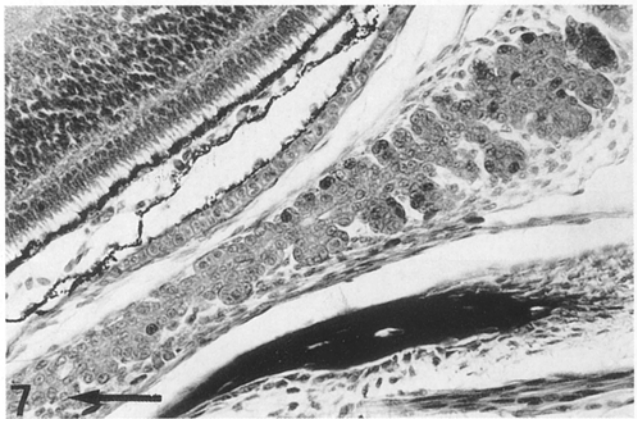
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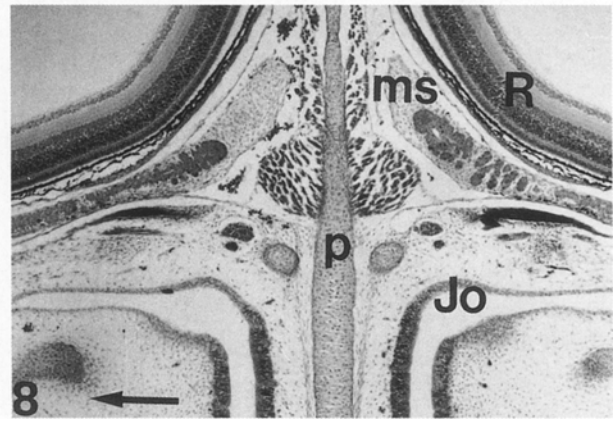
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For electron microscopy (EM), the heads were fixed for 2 h at 4° C in Karnowsky's fluid in phosphate buffer at pH 7.4 and post-fixed for 2 h at 4° C in 1% osmium tetroxide in the same buffer. After fixation, the samples were dehydrated through a series of ethyl alcohols and embedded in TAAB 812. Grids stained with 4% uranyl acetate followed by 1% lead citrate were examined with a Philips 301 transmission microscope.

At hatching, the HG and the LG of the lizards were first removed from the orbit and then processed as above.

Results

Histology and histochemistry

The anlage of the HG makes its first appearance on either side of the orbit in embryos at 22 days of development (stage 37; about 24 mm of total length). The appearance of this primordium is concomitant with the development of the nictitating membrane and immediately follows that of the eyelids (embryos of 21 days). The glandular primordium appears in the form of a short tubule projecting from the conjunctival epithelium at the medial corner of the orbit (Fig. 1). The mesenchymal cells surrounding the glandular blastema proliferate at a high rate and form a well-defined "sac", which corresponds to the area occupied later by the medial orbital glands (Fig. 2). At this stage the cells of the glandular primordium are weakly PAS- and AB/PAS-positive.

At 24 days of development (stage 39; about 28 mm of total length), the glandular blastema elongates in the form of a tube (Fig. 3). Prospective glandular cells, which face the mesenchyme directly, contact mesenchymal cells without a basal lamina separating these elements.

Fig. 1–11 Frontal sections of the whole head of lizard embryos. *Arrow* indicates medial versus lateral direction

Fig. 1 A 22-day embryo. The Harderian gland (HG) primordium (*arrowhead*) appears in the mesenchyme of the nictitating membrane projecting from the conjunctival epithelium (*E* eyelid, *NM* nictitating membrane). Mallory's trichrome. ×312

Fig. 2 A 22-day embryo. Medially to the eye, a symmetrical mesenchymal "sac" develops. It will be invaded later by anterior lacrimal gland (ALG) and HG lobules (*Jo* Jacobson's organ, *ms* mesenchymal "sac", *p* parasphenoid, *R* retina). Mallory's trichrome. ×50

Fig. 3 A 24-day embryo. The HG blastema (*arrowhead*) elongates from its point of origin. Mallory's trichrome. ×312

Fig. 4 A 26-day embryo. The HG blastema forms acini lacking a central lumen (*arrowheads*), surrounded by flat mesenchymal cells. Mallory's trichrome. ×312

Fig. 5 A 28-day embryo. The lobules increase in size (*arrowheads*). Mallory's trichrome. ×312

Fig. 6 A 28-day embryo. Some lobules (*arrowheads*) invade the preformed mesenchymal "sac". Mallory's trichrome. ×50

Fig. 7 A 36-day embryo. The HG lobules branch and connect to a central duct. Note the appearance of dark nuclei, which show a higher affinity for aniline blue. Mallory's trichrome. ×312

Fig. 8 A 36-day embryo. The glandular lobules push forward to the mesenchymal "sac". Mallory's trichrome. ×50

At the 26th day (stage 39; about 30 mm of total length) the glandular blastema forms acini at its distal end. Soon, each acinus extends and bifurcates, a process that is repeated several times. As they form, these acini, which still do not show a central lumen, elongate posteriorly into the mesenchymal "sac" situated in the medial part of the orbit (Fig. 4). Prospective glandular cells are weakly positive to the PAS and AB/PAS reactions. At this stage numerous small vessels appear in the stroma.

At the 28th day of development (stage 39; about 40 mm of total length) there is a clear increase in number and size of the acini. (Figs. 5, 6). These acini, which do not have a central lumen, invade to a greater extent the preformed mesenchymal "sac".

On the 36th day (stage 39; about 45 mm in length), as a result of continued cell proliferation, the acini branch and increase to a large extent throughout the mesenchymal parenchyma (Figs. 7, 8). Consequently, the mesenchymal stroma is progressively reduced and the acini flow together into a central duct. Prospective glandular cells are still weakly positive to PAS and AB/PAS reactions. Many nuclei show a marked affinity to aniline blue with the trichrome Mallory's stain (Fig. 7).

At the 41st day of development (stage 40; about 50 mm of total length) the process is complete; the lobules fill up the preformed mesenchymal "sac" (Fig. 9). The most medial part of the gland differentiates into the anterior lacrimal gland (ALG). At this stage a basal lamina, strongly PAS-positive, appears surrounding externally the contigous HG and the ALG. Unlike the HG, the glandular cells of the ALG become strongly PAS- and AB/PAS-positive (Fig. 9). At this stage the posterior lacrimal gland (PLG) can be seen in the posterior commissure of the eyelid (Fig. 10). It is firmly attached to the tissue of the orbital rim and consists of an invagination of the conjunctival epithelium. The inner part of the invagination organizes into acini, which are moderately PAS-positive.

From this stage (40; about 52 mm of total length) until hatching (about the 43rd day of development), the size of the HG remains almost constant, showing only a very slow increase. In contrast, the ALG and PLG continue to grow at a high rate so that, by the time of hatching, they are very well developed. The PLG becomes strongly PAS- and AB/PAS-positive (Fig. 11). Therefore, the highest rate of growth of the LG occurs between the 41st and 43rd day, while that of the HG occurs between the 36th and 41st day.

At day 8 after hatching (embryos of about 80 mm of total length), the HG acinar cells are filled by serous secretory granules, weakly AB- and AB/PAS-positive. Numerous melanocytes are seen scattered throughout the acinar cells. Also the LGs show the same features as those of the adult glands. Their mucous secretory granules are strongly PAS- and AB/PAS-positive.

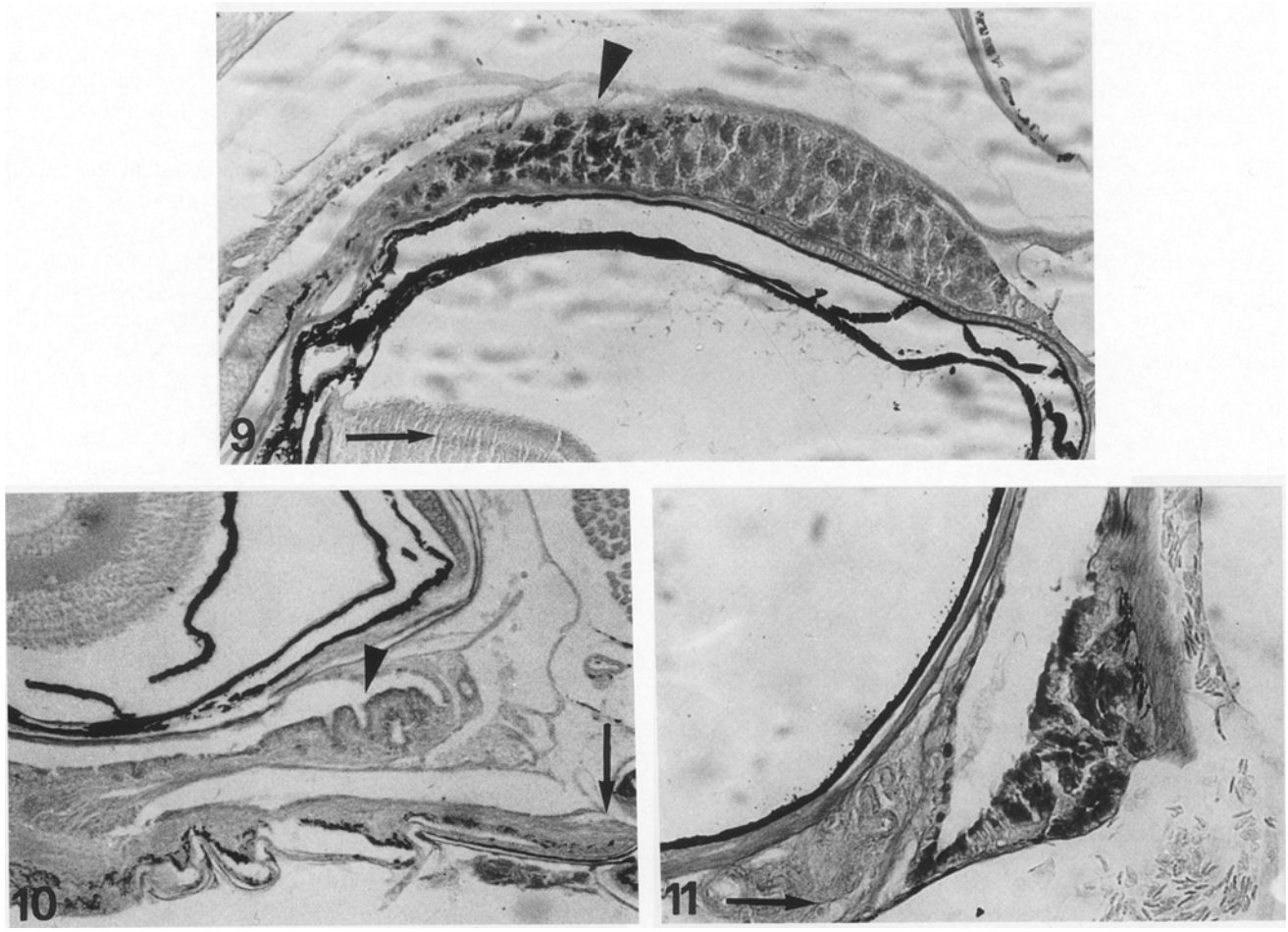


Fig. 9 A 41-day embryo. At the medial commissure of the eyelid, the HG lobules invade all the mesenchymal "sac". The most medial zone of the gland differentiates into the ALG which is strongly PAS- and Alcian-PAS-positive (*arrowhead*). PAS reaction. ×125

Fig. 10 A 41-day embryo. At the posterior commissure of the eyelid, the PLG (*arrowhead*) differentiates from the conjunctival epithelium. PAS reaction. ×125

Fig. 11 At hatching the PLG is composed of strongly PAS-positive acini. PAS reaction. ×500

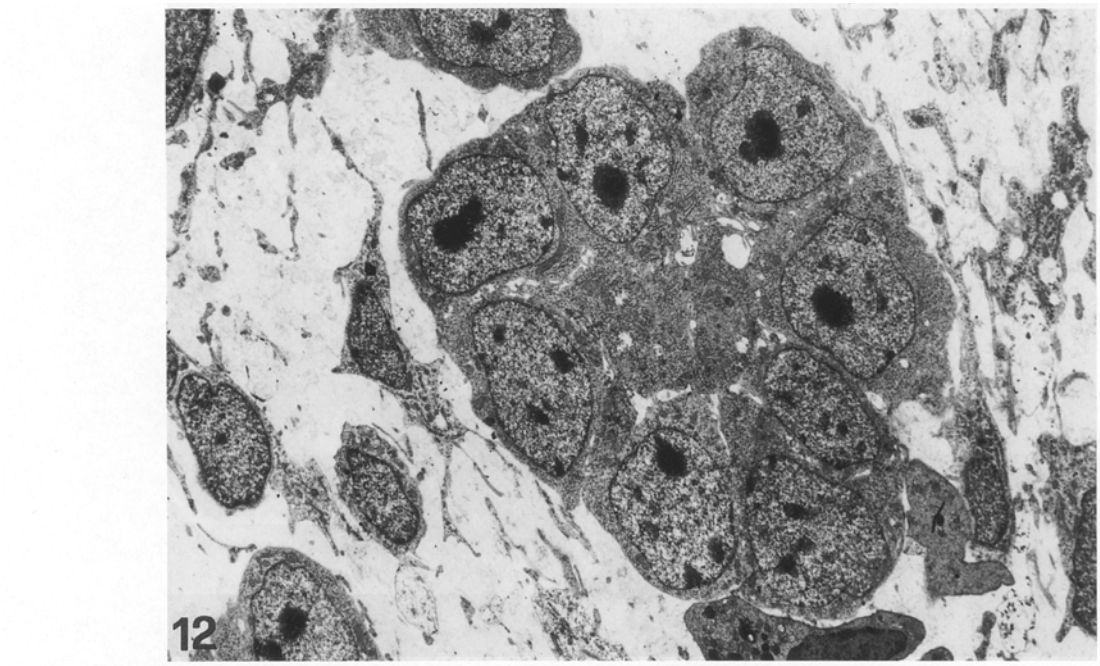
Ultrastructure

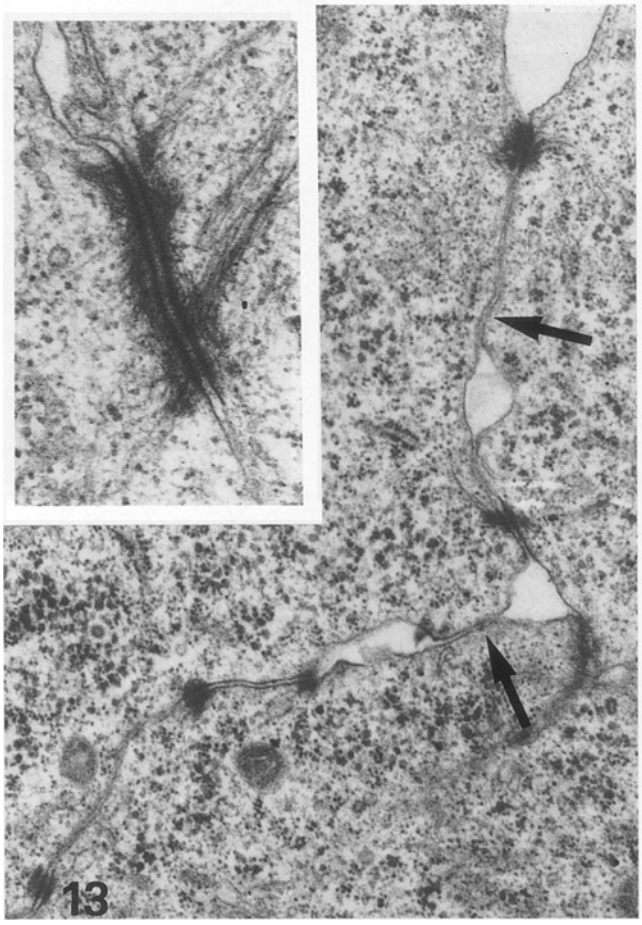
EM observations started at the 26th day of development (stage 39). At this stage prospective glandular cells appear aggregated in "rosette-like" structures in which lumina are not observed (Fig. 12). These cells are surrounded by mesenchymal cells without any evidence of a basal lamina. The nuclei show evident nucleoli, and the cytoplasm, devoid of secretory granules, contains free polyribosomes, numerous mitochondria and occasionally small cisterns of RER. The lateral border of the cells shows numerous interdigitations, while junctional complexes are frequently observed. These junctions are usually desmosomes and are found spaced at regular intervals along the membrane between the glandular cells (Fig. 13, inset). Occasionally such direct cell-cell contact had the typical appearance of a gap junction (Fig. 13). Similar cell-cell juxtapositions are seen frequently between glandular and mesenchymal cells (Fig. 14, inset).

In the embryos of 28 days (stage 39) the ultrastructural features of the glandular blastema remain similar to those of the previous stage. The first secretory granules appear at the 36th day of development (stage 39). They have a heterogeneous aspect with an electron-dense component (Fig. 15, inset). The cells also contain numerous mitochondria, RER and microvilli on the apical surface that surrounds narrow lumina.

At hatching, the acinar cells of the HG show a gradual increase in cytoplasmic volume and a very well-developed RER. Secretory granules in various stages of maturation are visible (Fig. 16). Most of these show in addition a component of lower density formed by randomly arranged granules (Fig. 16, inset). Sloughing of cells can often be seen within the acini, and this event leads to the enlargement of the lumen (Fig. 16).

At hatching, the intermediate zone of the HG is also distinguishable with the EM. This zone, which is not visible in histological preparations, appears between the ALG and the HG; it is formed by cells that contain both mucous and serous granules (Fig. 17). At the same time, the ALG and PLG contain moderately electron-dense granules that have not yet gained the aspect seen in the adult lacrimal cells (Fig. 18). Several cells are devoid of secretory granules.





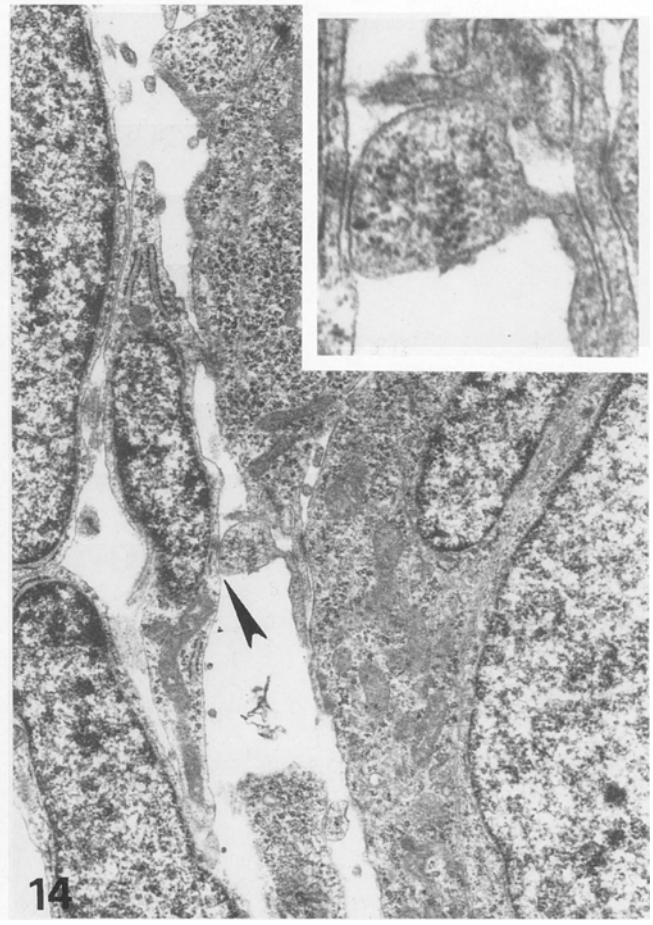


Fig. 12 Electronmicrograph of the HG primordium of a 26-day embryo. The prospective glandular cells are arranged in a "rosette-like" structure. ×2800

Fig. 13 Electronmicrograph of the HG primordium of a 26-day embryo showing junctional complexes (desmosomes) spaced at regular intervals between glandular cells. Occasionally gap junction are seen (*arrows*). ×12,000. *Inset* higher magnification of a desmosome. ×20,000

Fig. 14 Electronmicrograph of the HG primordium of a 26-day embryo showing a focal contact (*arrowhead*) between a glandular cell and a mesenchymal cell. ×4,400. *Inset* higher magnification of the focal contact. ×30,000

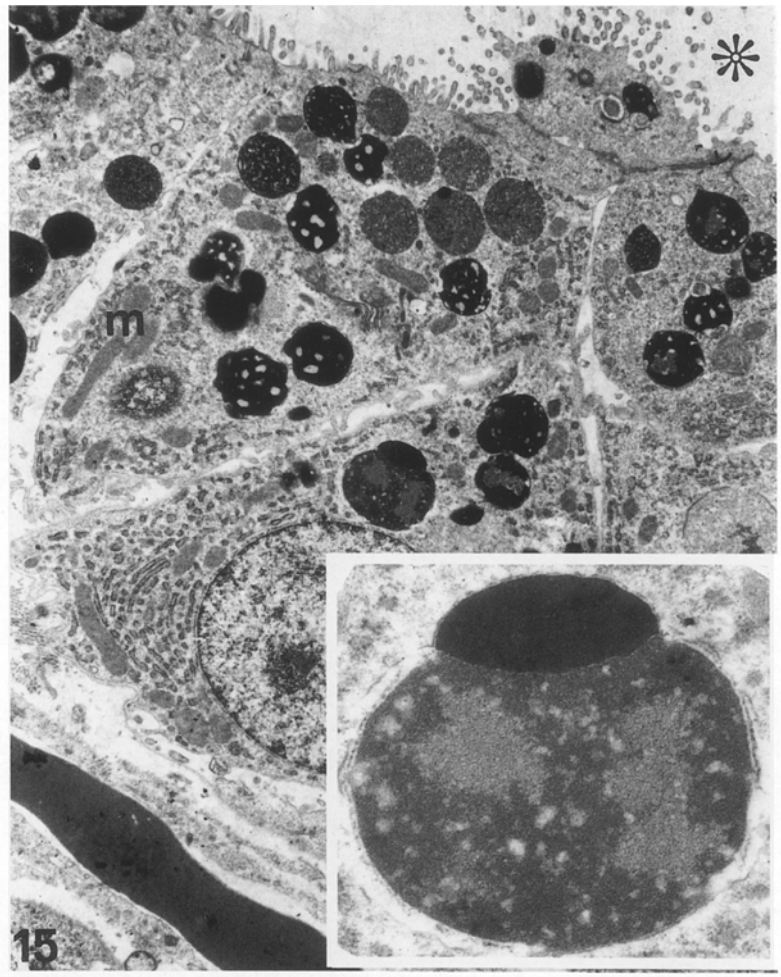


Fig. 15 Electronmicrograph of a 36-day embryo HG showing the appearance of the secretory granules. They have a heterogeneous aspect with an electron-dense component. The glandular cells contain numerous elongated mitochondria (*m*), abundant RER and microvilli extending into the acinar lumina (*asterisk*). ×3,000. *Inset* higher magnification of the secretory granule. ×20,000

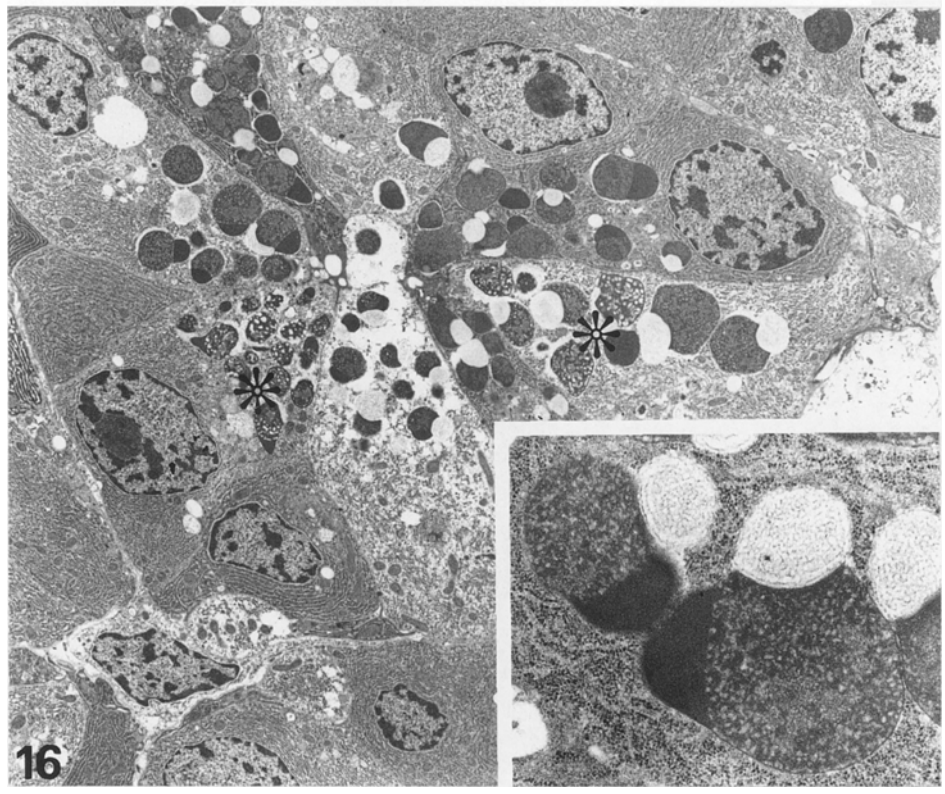


Fig. 16 Electronmicrograph of an HG acinus at hatching, showing granules in various stages of maturation. ×2,800. *Inset* higher magnification of the granules. The less dense component shows randomly arranged granules. ×13,000

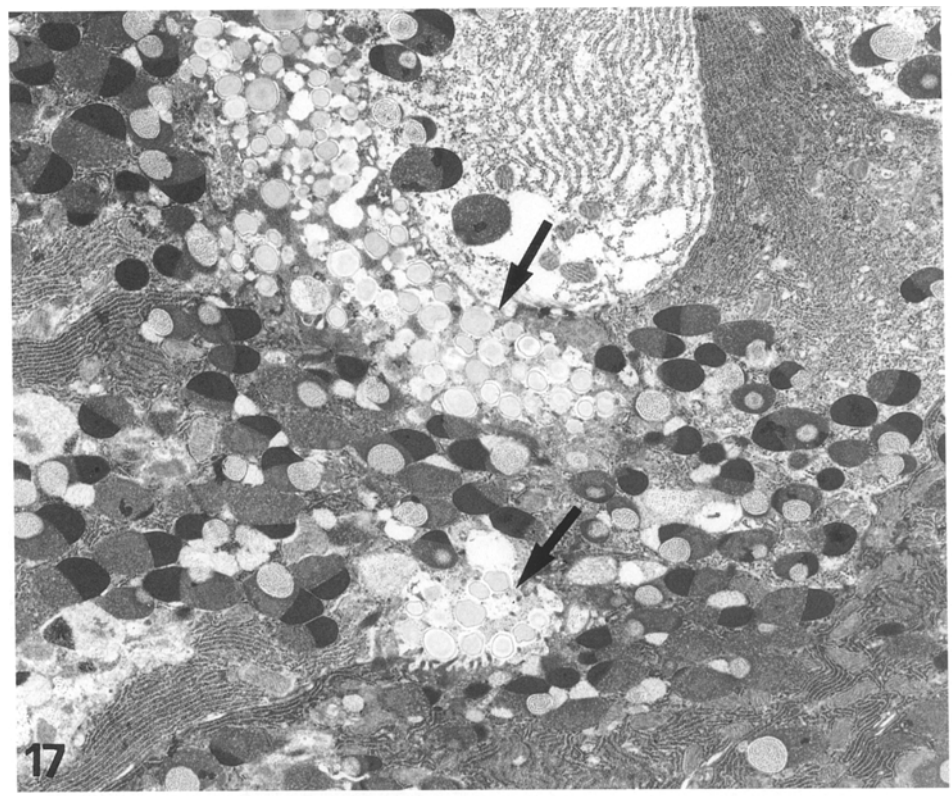


Fig. 17 Electronmicrograph of the intermediate zone of the HG at hatching. The acinar cells contain both serous and mucous secretory granules (*arrows*). ×4,600

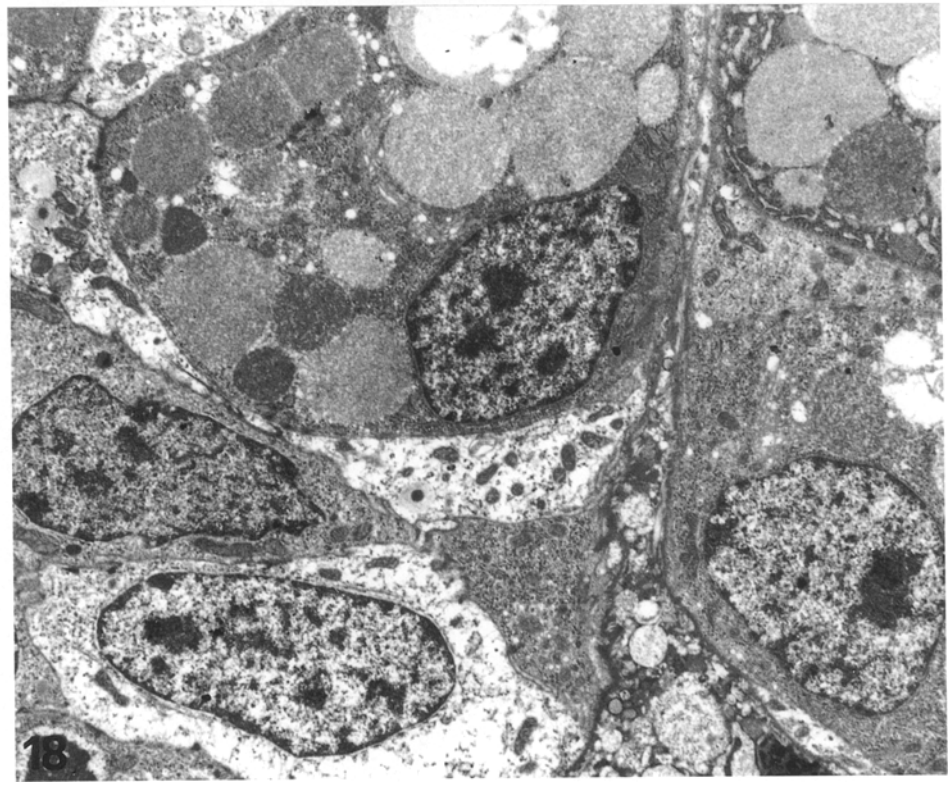


Fig. 18 Electronmicrograph of the ALG at hatching. Only few glandular cells contain mucous secretory granules. ×3,000

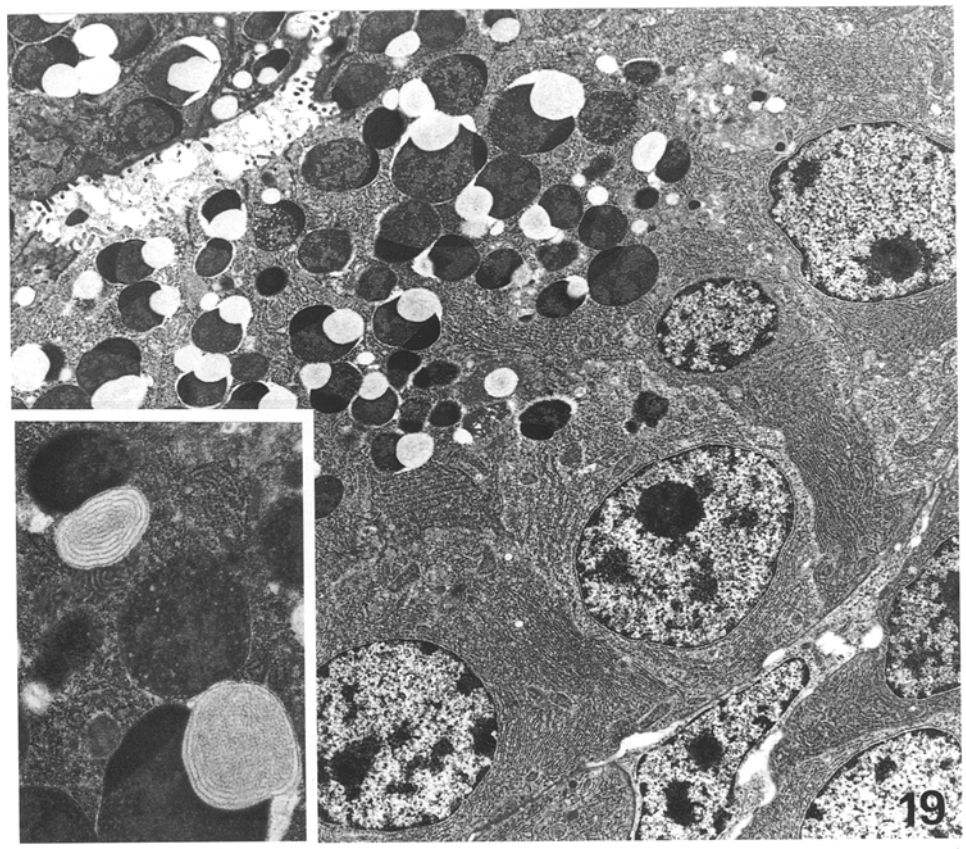


Fig. 19 Electronmicrograph of a HG acinus with an enlarged lumen, 8 days after hatching. Most of the glandular cells contain numerous "special secretory granules". ×2,800. *Inset* higher magnification of the granules. The less dense component shows the laminar structure of the adult secretory granules. ×12,000

In 8-day-old lizards, the maturation of the HG secretory cells is complete (Fig. 19). The mature secretory granules differ from those of the previous stage, because the component of lower density is formed by granules arranged in concentric rings (Fig. 19, inset). The acini have large lumina, and the lacrimal glands also show the same characteristics as those of the adult glands. Their mucous granules are non-homogeneous and irregular in shape.

Discussion

Very few studies have concentrated on the embryological development of the LG and HG, and of these none have been carried out at the EM level. Sakai (1981), reported two major theories to explain the embryological origin of the LG and the HG. The first is the "single gland" or "migration" theory proposed by Wiedersheim in 1908. It suggests that the LG and the HG might originate from a single primordium situated in the lower lid. The HG would be formed from the nasal region of this precursor, while the remaining part moves to the upper lid and becomes the lacrimal gland.

The second, "two glands" theory, proposes the existence of two distinct primordia, the LG in the outer and the HG in the inner canthus and assumes that these arose from a common precursor gland during the course of vertebrate evolution. Sakai (1981) concludes that the mammalian HG may not be homologous with that of the lower vertebrates, and that in the phylogeny of the mammalian HG it appears more probable that this evolved from the LG of a primitive ancestral mammal.

Michael et al.(1988) found that the mouse HG develops on the 16th day of prenatal life, while the LG appears on the 7th day of postnatal life, and no hint is given concerning a possible common precursor. However, Shirama and Hokano (1991) considered that the epithelium of the mouse HG differentiates only at the 7th day after birth.

Our observations seem to be consistent with the "single gland" theory. In fact the ALG and the HG of the lizard share a common embryological origin from the conjunctival epithelium at the medial corner of the orbit, while the PLG originates from the conjunctival epithelium in the posterior commissure of the eyelid. The anlage of the medial orbital glands (ALG and HG) may be recognized in embryos from the 22nd day of development. This event coincides with the appearance of the nictitating membrane. The contemporaneousness of these two events has been shown during metamorphosis in some species of anuran amphibia. Shirama et al. (1982) showed that in the frog, *Rana japonica* the HG appeared at the late prometamorphic stage, and in the toads, *Bufo bufo japonicus* and *Xenopus laevis* around the climax. Also, in the frog *Rana esculenta* and the toad *Bufo viridis* the HG developed during the late prometamorphic stage (Chieffi Baccari et al. 1990a). However, in the mouse Michael et al.(1988) found that the HG develops

on the 16th day of prenatal life, while the third eyelid develops after birth.

In the lizard embryo, the growth of the glandular blastema, after its initial appearance in the form of an evagination of the conjunctival epithelium, continues with the formation of lobules which move posteriorly into a preformed mesenchymal "sac" (26th, 28th days; stage 39). At this time, the prospective glandular cells proliferate and organize themselves into "rosette-like" arrangements that do not have central lumina. They are undifferentiated and show numerous cell junctions, desmosomes and gap junctions. These junctional complexes are very numerous only during the first stages of glandular development. It is well known that from the outset, the cells of the embryos are not only bound together mechanically by desmosomes, but also coupled by gap junctions through which ions and other small molecules can pass. Although the significance of the gap junctions during embryogenesis is still unclear, various hypotheses have been suggested to explain their function. For example, the shape changes associated with rolling up into a tube during embryogenesis might well be coordinated by small metabolites and ions via gap junctions. However, in early mouse embryos, metabolic coupling between cells is most probably important before the blood circulatory system has developed sufficiently to convey nutrients. In some cases cells are known to couple transiently during development. It is possible that this is one mechanism whereby cells recognize each other in order to interact during development. According to this purely hypothetical view, cells could sample each other's intercellular environments by promiscuous coupling, terminating the interactions with cells that do not "taste" right (see Bard 1990).

The glandular blastema grows rapidly until the 36th day of development, at which stage it appears as a branched structure. Differentiated cells become visible, showing various cisterns of RER and serous granules. These granules resemble the "special secretory granules" described in the adult HG (Chieffi Baccari et al. 1990b). They have been described in this way because of their strikingly unusual structure, consisting of three closely associated components that differ either in density or in form.When they appear on the 36th day, these granules differ from those of the adult HG as they lack the less dense component, which appears for the first time only at hatching. However, only 8 days after hatching the secretory granules definitively acquire the adult characteristics.

The onset of secretory activity at the 36th day is marked by the presence of "blue nuclei" in numerous glandular cells. This unusual staining of the nuclei, which normally stain orange with the Mallory trichrome method, has been correlated in various vertebrate tissues with an increased rate of RNA synthesis (Chieffi Baccari et al. 1992a, b).

Owing to its progressive and continuous growth, the glandular parenchyma completely invades the mesenchymal "sac" before hatching (41st day of development). Only at this stage do the ALG and PLG differentiate. The contemporary differentiation of the anterior and posterior LGs is noteworthy.

The underlying reasons why, during development, the first cells that migrate into the mesenchymal "sac" differentiate into HG cells, while the last to migrate differentiate into LG cells are not known. Although there is circumstancial evidence suggesting that the initiation of morphogenesis is under the autonomous control of the participating cells (see Bard 1990), there is now increasing evidence of a mesenchymal influence on secondary induction, i.e. of the interactions between the mesenchyme and the epithelia derived from ectoderm or endoderm. This is typical of a large class of organs comprising a mesenchymal mass, like the mesenchymal "sac" of the lizard HG, in which is embedded an epithelial tube that branches repetitively, and whose branches end in lobules. In this case, the key molecule produced by the mesenchyme is hyaluronidase, which initiates and probably maintains the epithelial morphogenesis in the "on" state (see Bernfield et al. 1984).

Although the role of the mesenchyme in development of the orbital glands can only be postulated from this study, the fact that junctional complexes exist between epithelial cells and mesenchymal cells strengthens this hypothesis. Studies of cells in culture have described regions of contact as sites of adhesion of the cell to specific extracellular matrix proteins, such as fibronectin and proteoglycan (Burridge and Fath 1989). These attach to extracellular matrix receptors in the plasma membrane, which in turn attach to the cytoskeleton (Yurechenco and Schnittny 1990). In this way the mesenchymal elements would influence the overlying epithelial cell structure and function.

Spatial and temporal differences exist, of course, during the secondary induction of the medial orbital gland of the lizard. The inductive stimulus (or stimuli) responsible for the initial differentiation of the HG must be different from whatever induces differentiation of the anterior and posterior LGs at a later stage. Furthermore, a gradient of concentration of the inductive substance(s) can be envisaged, since an intermediate zone between the HG and the ALG exists, composed of mixed glandular cells containing both mucous and serous secretory granules.

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