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Yuzo Ogawa · Shinobu Yamauchi · Akio Ohnishi
Reiko Ito · Naokuni Ijuhin

Immunohistochemistry of myoepithelial cells during development of the rat salivary glands

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Abstract Using a battery of monoclonal antibodies specific for rat proteins, immunohistochemistry was carried out on the developing myoepithelial cells (MECs) of the rat major salivary glands. The proteins examined were α-smooth muscle actin (αSMA), h1-calponin (calponin), keratin 14 (K14), β subunit of S-100 protein (S-100β), vimentin and glial fibrillary acidic protein (GFAP). The MECs exhibited immunoreactivity for αSMA, calponin and K14, but not that for S-100β, vimentin and GFAP. Immunoreactivity for αSMA appeared in the MECs from the time when the microfilaments were initially deposited in these cells, i.e., at 20 days in utero in the sublingual and submandibular glands and at birth in the parotid gland. Calponin immunoreactivity was seen 1 day earlier than αSMA. The appearance was almost at the same time as the onset of the MEC differentiation in each gland. A small number of the MECs expressed weak K14 immunoreactivity from the time when the acinus-intercalated duct structure was established, i.e., at 21 days in utero in the sublingual gland, at 5 days after birth in the perotid gland and after 5 weeks post-natally in the submandibular gland. In addition, K14 immunoreactivity was observed in the basal cells of the striated and excretory ducts. The first appearance of K14 in these cells again coincided with the emergence of the duct system in each gland, i.e., at 20 days in utero in the sublingual gland, at 21 days in utero in the submandibular gland and at 3 days after birth in the parotid gland. Finally, the MECs in all the glands were found to redistribute as the acini matured. As the acini grew rapidly during the weaning period in the parotid and the sublingual glands, the MECs ceased to surround the acini. Thereafter, they disappeared from the acini in the parotid gland, whereas they reappeared in the sublingual gland. In the submandibular gland, the MECs were confined to the terminal tubules until 4 weeks after birth. Thereafter, the acini were established and invested by the MECs. In conclusion, immunohistochemistry of calponin and αSMA is a useful tool for identification of the MEC during its earliest differentiation, which has hitherto been possible only electron microscopically. In addition, it is suggested that the MEC is heterogeneous and the functionally differentiated MEC appears after weaning around acini of the mucous and seromucous glands.

Key words Alpha-smooth muscle actin · Calponin · Keratin · S-100 protein · Vimentin · Glial fibrillary acidic protein

Y. Ogawa (✉) · S. Yamauchi · A. Ohnishi · R. Ito · N. Ijuhin
Department of Oral Pathology, Osaka University
Faculty of Dentistry, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan
e-mail: ogawa@dent.osaka-u.ac.jp
Tel.: +81-6-6879-2892, Fax: +81-6-6879-2895

Introduction

Myoepithelial cells (MECs) are found in the secretory units of many mammalian exocrine glands such as mammary, sweat, lacrimal and salivary glands. They are interposed between the secretory cells and the basal lamina, contain keratin intermediate filaments (Franke et al. 1980) and are, therefore, thought to be epithelial in origin. On the other hand, MECs contain a large number of microfilaments or myofilaments, representing massive expression of contractile proteins such as actin, myosin, calponin and caldesmon (Drenckhahn et al. 1977; Longtine et al. 1985; Gugliotta et al. 1988; Lazard et al. 1993; Deugnier et al. 1995). These proteins have been considered to be specific markers for smooth muscle cells. To date, however, there have been few comprehensive studies addressing the question of the expression of the epithelium- and smooth muscle-specific proteins by developing MECs.

A number of salivary gland neoplasms have prominent component cells that are named “modified or neoplastic MECs”. However, it has not yet been established that they actually originate from MECs because they often lack epithelial and smooth muscle proteins that are normally expressed by MECs. Moreover, the “neoplastic MECs” express non-epithelial, non-smooth muscle proteins such as S-100 protein, vimentin and glial fibrillary acidic protein

(GFAP; Ellis and Auclair 1996). To gain an insight into the histogenesis and morphogenesis of salivary gland tumors, it is worthwhile to determine whether the developing MECs express the protein markers characteristic of the "neoplastic MECs". Although there have been investigations into the developmental changes in S-100 protein, vimentin and GFAP expressions in human salivery gland (Gustafsson et al. 1988; Lee et al. 1993; Adi et al. 1994; Chisholm and Adi 1995), systematic time course study was difficult with human materials and these descriptions of the differentiation of the MEC were sketchy (Redman 1994).

In this study, we examine the rat major salivary glands for developmental changes in cytochemically demonstrable immunoreactivity for α-smooth muscle actin (αSMA), h1-calponin (calponin), keratin 14 (K14), β subunit of S-100 protein (S-100β), vimentin and GFAP. The rat was used because thorough morphological analyses have been made on the development of major salivary glands both at light- and electron-microscopic levels (Line and Archer 1972; Cutler and Chaudhry 1973a; Redman and Ball 1979; Redman et al. 1980; Norberg et al. 1996).

Materials and methods

Male rats older than 1 week of age and pregnant rats of the Sprague-Dawley strain were purchased from Nihon Dohbutsu (Osaka, Japan). Embryos at 18, 19, 20 and 21 embryonic days, pups at 0, 1, 3 and 5 days after delivery and 1, 2, 3, 4 and 5 weeks, and adult rats (4–6 months) were killed and examined. All experiments were reviewed and approved by the Osaka University Faculty of Dentistry Intramural Animal Use and Care Committee prior to the study. Each age group consisted of at least three animals. For postnatal examination, only male rats were used. The monoclonal antibodies used for immunohistochemistry, their dilutions and their sources are listed in Table 1.

Tissue preparation

Embryos were taken from chloral hydrate (20 mg/100 g b.w.)-anesthetized mothers, and the head-and-necks were removed. They were fixed in methacarn (Puchtler et al. 1970; Mitchell et al. 1985) for 36 h or 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4° C. Postnatal rats were anesthetized with chloral hydrate, and the submandibular, major sublingual and parotid glands were removed. They were cut into small tissue blocks and fixed in methacarn for 36 h. Some postnatal animals were perfused through the left ventricle with the paraformaldehyde fixative. The glands were removed and cut into small tissue blocks. They were further fixed in the same fixative overnight at 4° C.

After fixation in methacarn, the head-and-necks and the salivary tissue blocks were processed according to the method of Puchtler et al. (1970) and embedded in paraffin. Paraformaldehyde-fixed materials were processed according to the method described previously (Ogawa et al. 1992; 1995) and frozen in liquid nitrogen. Paraffin (2–4 μm) and frozen (6–12 μm) sections were cut and mounted on silane-coated glass slides. Paraffin sections were deparaffinized, re-hydrated and immersed in deionized water. Frozen sections were air-dried. Both paraffin and frozen sections were then immersed in 0.01 M phosphate-buffered 0.145 M NaCl (pH 7.2; PBS) and used for immunohistochemistry.

Light-microscopical immunohistochemistry

Sections were incubated for 30 min with 0.3% H_2O_2 to block endogenous peroxidase, and then for 30 min with 10% normal rabbit serum in PBS containing 1% bovine serum albumin (PBS-BSA) to block nonspecific binding. After the serum was wiped away (without washing), the sections were incubated overnight at 4°C with one of the primary antibodies, diluted with PBS-BSA, then for 60 min with biotinylated rabbit anti-mouse immunoglobulins (1:500 in PBS-BSA containing 0.5% normal rat serum: DAKO A/S, Glostrup, Denmark), and finally for 30 min with streptavidin-biotinylated horseradish peroxidase reagent (1:100 in PBS; DAKO A/S). Incubation for 3 min with 3,3′-diaminobenzidine tetrahydrochloride (DAB)-H_2O_2 solution was carried out to visualize the immunoreaction sites. All the above steps were followed by at least three 10-min washes with PBS at 4°C. Sections were counterstained with methyl green, dehydrated, and coverslipped with Permount.

Electron-microscopical immunohistochemistry

Frozen sections were used for electron-microscopical immunohistochemistry of S-100β, vimentin and GFAP. The indirect immunoperoxidase method was carried out as described previously (Ogawa et al. 1992, 1995). Sections were treated with normal rabbit serum. The serum was wiped away and, without washing, the sections were incubated with the primary antibody overnight at 4° C. After inactivation of endogenous peroxidase with H_2O_2 solution, the sections were incubated overnight at 4° C with peroxidase-conjugated rabbit anti-mouse IgG (1:200: DAKO A/S). They were re-fixed with 2.5% glutaraldehyde in PBS for 10 min at 4° C, and then serially incubated with DAB (containing 1% dimethyl sulfoxide) and DAB-H_2O_2 solutions for 30 min and 5 min, respectively. The sections were washed thoroughly with PBS at 4° C after each step. After the final wash, the sections were treated with 1% O_SO_4 reduced with 1% $K_4Fe(CN)_6$ in 0.1 M phosphate buffer (pH 7.4) for 45 min at 4° C. They were dehydrated and embedded in an epoxy resin (Epok 812; Oken Shoji, Tokyo, Japan). Ultrathin sections were examined without counterstaining, using a JEOL 2000 EX electron microscope.

Controls and miscellaneous protocols

Negative controls for immunostaining were performed by substituting the primary antibodies with PBS and normal mouse IgG (Miles Scientific, Naperville, Ill., USA).

For the calponin stain, the above procedure yielded extensive nuclear staining of a wide variaty of featal cells including glandular mesenchymal and epithelial cells, the skeletal muscle fibers

Table 1 Monoclonal antibodies used for immunohistochemistry

Antibody	Specificity	Dilution	Source
1A4	α-smooth muscle actin	1:1000	Dako A/S, Glostrup, Denmark
hCP[a]	H1-calponin	1:2000	Sigma Chemical Co., St. Louis, Mo., USA
LL002[a]	Keratin 14	1:20	Ylem, Roma, Italy
SH-B1[b]	β-chain of S-100 protein	1:1000	Sigma
V9	Vimentin	1:500	Dako A/S
6F2	Glial fibrillary acidic protein	1:2000	Dako A/S

[a] Antibody usable only for methacarn-fixed, paraffin-embedded material
[b] Antibody usable only for paraformaldehyde-fixed, frozen material

and the cells of the central nervous system. Such nuclear stain was completely suppressed when the buffer system (0.145 M NaCl) was substituted with a hypertonic PBS (0.5–1.0 M). On the other hand cytoplasmic stain of the adult vascular smooth muscle cells, as used for the positive control, was not affected by the hypertonic PBS. Therefore, we limit our description to the observation on the calponin stain resistant to the hypertonic buffer system.

The keratin stain yielded only a weak immunoreaction in small number of MECs (see results). To exclude a possibility that methacarn fixation interfered with the immunoreactivity, some materials were processed as un-fixed frozen sections, but the result was not improved (data not shown).

Results

The rat MECs expressed immunoreactivity for αSMA, calponin and K14. The immunoreactivity for αSMA, calponin and K14 by the MECs in each gland at each developmental stage is summarized in Tables 2–4. S-100β, vimentin and GFAP were not detected in the MECs throughout the examined developmental period.

The morphology of developing rat salivary glands has been thoroughly described by several authors (Jacoby and Leeson 1959; Leeson and Booth 1961; Redman and Sreebny 1970; Redman and Sreebny 1971; Cutler and Chaudhry 1974; Redman and Ball 1978) and will not be detailed.

Parotid glands (Table 2)

At 18 days in utero, the developing salivery glands consisted of cellular cords terminating in clusters of cells called terminal buds (Redman et al. 1980). Lumenization had already occurred in these structures both in the submandibular and the sublingual glands but not the parotid gland. No positive staining was observed in any cell type for any antibody until 21 days in utero.

At 21 days in utero, lumenization was seen giving rise to duct formation in the cellular cords of the parotid gland. The differentiating MECs with weak cytoplasmic calponin immunostaining were sparsely scattered in the outermost layer of the terminal buds and the small ducts adjacent to them (Fig. 1a; Table 2). During the following few days, there was a rapid increase both in the staining intensity and the number of stained cells. Consequently, the calponin-positive MECs occupied a large part of the outer layer of the terminal buds and small ducts (Fig. 1c). At birth, some of the MECs acquired αSMA immunoreactivity (Table 2), and on the following day almost all the MECs acquired both calponin and αSMA immunoreactivities. The sequence of αSMA acquisition by the calponin-positive MECs was clearly seen in an alternate series of sections stained for calponin and αSMA (Fig. 1b–d; see also Figs. 2b–e, 3b–e). From 5 days after birth, a small number of MECs were stained weakly by anti-K14 antibody (Fig. 1e; Table 2). By about 1 week, the terminal buds and the small ducts had differentiated into acini and intercalated ducts, respectively (Table 2). These structures consisted of two layers of cells, i.e., an inner layer of pyramidal or columnar cells around the lumen and an outer layer of somewhat flattened MECs that were positive for calponin, αSMA and occasionally K14 (Fig. 1f). After 2 weeks, there was a marked increase in the size of the acini and acinar cells, and the MECs showed an elongated profile (Fig. 1g). By 3 weeks, the number of the MECs had decreased around the acini (Table 2). A 4 weeks, when the developing gland assumed a morpholo-

Table 2 Relative intensity of expression of smooth muscle and epithelial markers by myoepithelial cells in the developing parotid gland

Age	αSMA[a]		H1-calponin		Keratin 14	
Prenatal	Terminal bud+small duct		Terminal bud+small duct		Terminal bud+small duct	
18 Days in utero	0		0		0	
19 Days in utero	0		0		0	
20 Days in utero	0		0		0	
21 Days in utero	0		±		0	
Postnatal						
0 Day	+		+		0	
1 Day	++		++		0	
	Immature acinus	Small duct	Immature acinus	Small duct	Immature actinus	Small duct
3 Days	++	++	++	++	0	0
5 Days	++	++	++	++	+	+
	Acinus	Intercalated duct	Acinus	Intercalated duct	Acinus	Intercalated duct
1 Week	++	++	++	++	+	+
2 Weeks	++	++	++	++	+	+
3 Weeks	++[b]	++	++[b]	++	+[b]	+
4 Weeks	0	++	0	++	0	+
5 Weeks	0	++	0	++	0	+
Adult	0	++	0	++	0	+

[a] αSMA, α-smooth muscle actin
[b] The number of the positive cells is smaller than in the previous stage

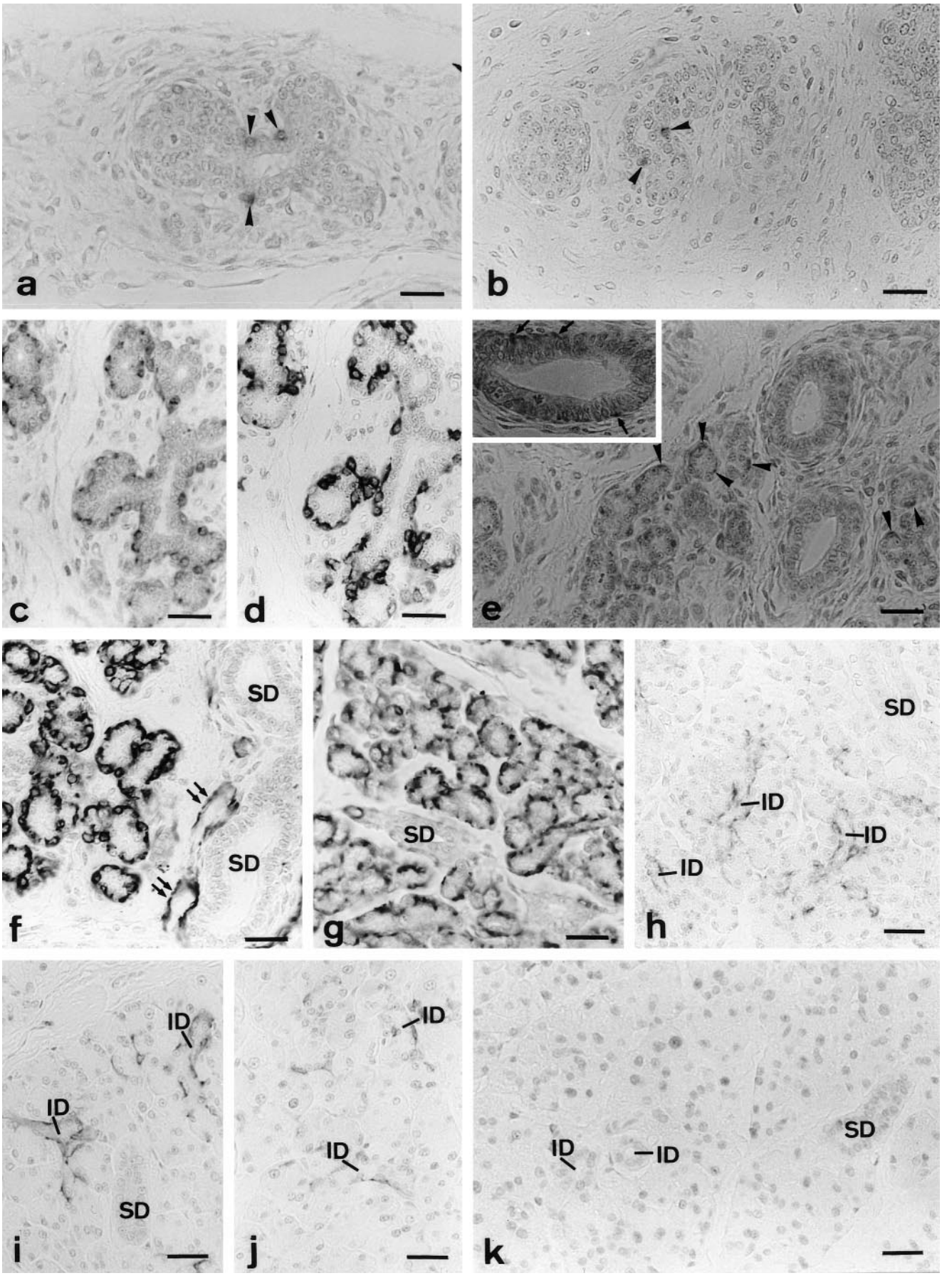
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Table 3 Relative intensity of expression of smooth muscle and epithelial markers by myoepithelial cells in the developing submandibular gland

Age	αSMA[a]		H1-calponin		Keratin 14	
Prenatal	Terminal tubule		Terminal tubule		Terminal tubule	
18 Days in utero	0		0		0	
19 Days in utero	0		±		0	
20 Days in utero	+		+		0	
21 Days in utero	++		++		0	
Postnatal						
0 Day	++		++		0	
1 Day	++		++		0	
3 Days	++		++		0	
5 Days	++		++		0	
	Immature actinus	Terminal tubule	Immature acinus	Terminal tubule	Immature acinus	Terminal tubule
1 Weeks	0	++	0	++	0	0
2 Weeks	0	++	0	++	0	0
3 Weeks	0	++	0	++	0	0
4 Weeks	0	++	0	++	0	0
	Acinus	Intercalated duct	Acinus	Intercalated duct	Acinus	Intercalated duct
5 Weeks	++[b]	++	++[b]	++	0	0
Adult	++	++	++	++	+	+

[a] αSMA, α-smooth muscle actin
[b] The number of the positive cells is smaller than in the following stage

gy almost indistinguishable from the adult one, the MECs no longer embraced the acini (Fig. 1h; Table 2). Thereafter, the MECs positive for calponin, αSMA and occasionally K14 were present as narrow bands around and along the intercalated ducts (Fig. 1i, j).

Submandibular gland (Table 3)

In the submandibular gland, the first sign of MEC differentiation was detected at 19 days in utero when lumenization in the terminal buds had already occurred and given rise to elongated, two-cell-layered structures – the terminal tubules (Jacoby and Leeson 1959). The differentiating MECs were recognized as cells with weak calponin immunostaining. They were scattered in the outer layer of the terminal tubules (Fig. 2 a; Table 3). The calponin-positive MECs rapidly increased, thereafter, both in staining intensity and number of the stained cells. In addition, some MECs acquired αSMA immunoreactivity at 20 days in utero (Fig. 2b, c; Table 3). By birth, all the calponin-positive MECs appeared to have acquired αSMA immunoreactivity (Fig. 2d, e).

Unlike the parotid gland, the submandibular gland had a MEC lining covering the intercalated duct as well as the acini in the adulthood. However, maturation of acini progressed much more slowly than in the tubular system. MEC lining was confined to the terminal tubules in neonates (Fig. 2f; Table 3). At 4 weeks the intercalated duct was the only structure invested by the MECs (Fig. 2g), while the acini still lacked the lining. The acinar MEC lining first appeared between 4 and 5 weeks, but was much less extensive than in the adult (Fig. 2h; Table 3). K14 immunoreactivity was not detected in MECs at or earlier than 5 weeks (Table 3). In the adult submandibular gland, both the intercalated ducts and the acini were invested by the MECs, which occasionally expressed weak K14 immunoreactivity (Fig. 2i–k) in addition to calpoint and αSMA.

Sublingual gland (Table 4)

The MEC differentiation and its expression of calponin and αSMA in the sublingual gland showed a time course

Fig. 1a–k Immunoperoxidase histochemistry of rat parotid glands fixed in methacarn. Calponin (**a**, **c**, **i**); αSMA (**b**, **d**, **f–h**); K14 (**e**, **j**). Sections were counterstained with methyl green. **a** 21 Days in utero. MECs weakly positive for calponin are seen sporadically at the outer layer of terminal buds and small ducts adjacent to them (*arrowheads*). **b** Newborn. For the first time, MECs express αSMA immunoreactivity (*arrowheads*). **c**, **d** 1 Day postnatally. Serial sections. Calponin-positive MECs occupy large parts of the outer layer of terminal buds and small ducts (**c**). Almost all of these cells express αSMA immunoreactivity (**d**). **e** 1 Week. A small number of MECs express weak K14 immunoreactivity (*arrowheads*). Basal cells of striated ducts also express weak K14 immunoreactivity (*arrows* in *inset*). **f** 1 Week. Terminal buds and small ducts have differentiated into acini and intercalated ducts, respectively. Outer layer of these structures consist of αSMA-positive MECs. Note αSMA-positive vascular walls (*double arrows*). **g** 2 Weeks. As acini and acinar cells increase, the gland becomes compact. MECs still surround both acini and intercalated ducts. **h** 4 Weeks. Developing gland is almost indistinguishable from adult one. MECs almost exclusively enclose intercalated ducts. **i**, **j** Adult. MECs are seen along intercalated ducts. They are positive for calponin (**i**), αSMA and occasionally K14 (**j**). **k** Adult. Negative control – normal mouse IgG was substituted for the primary antibody (*SD* striated duct, *ID* intercalated duct). *Bars* 25 μm (×320)

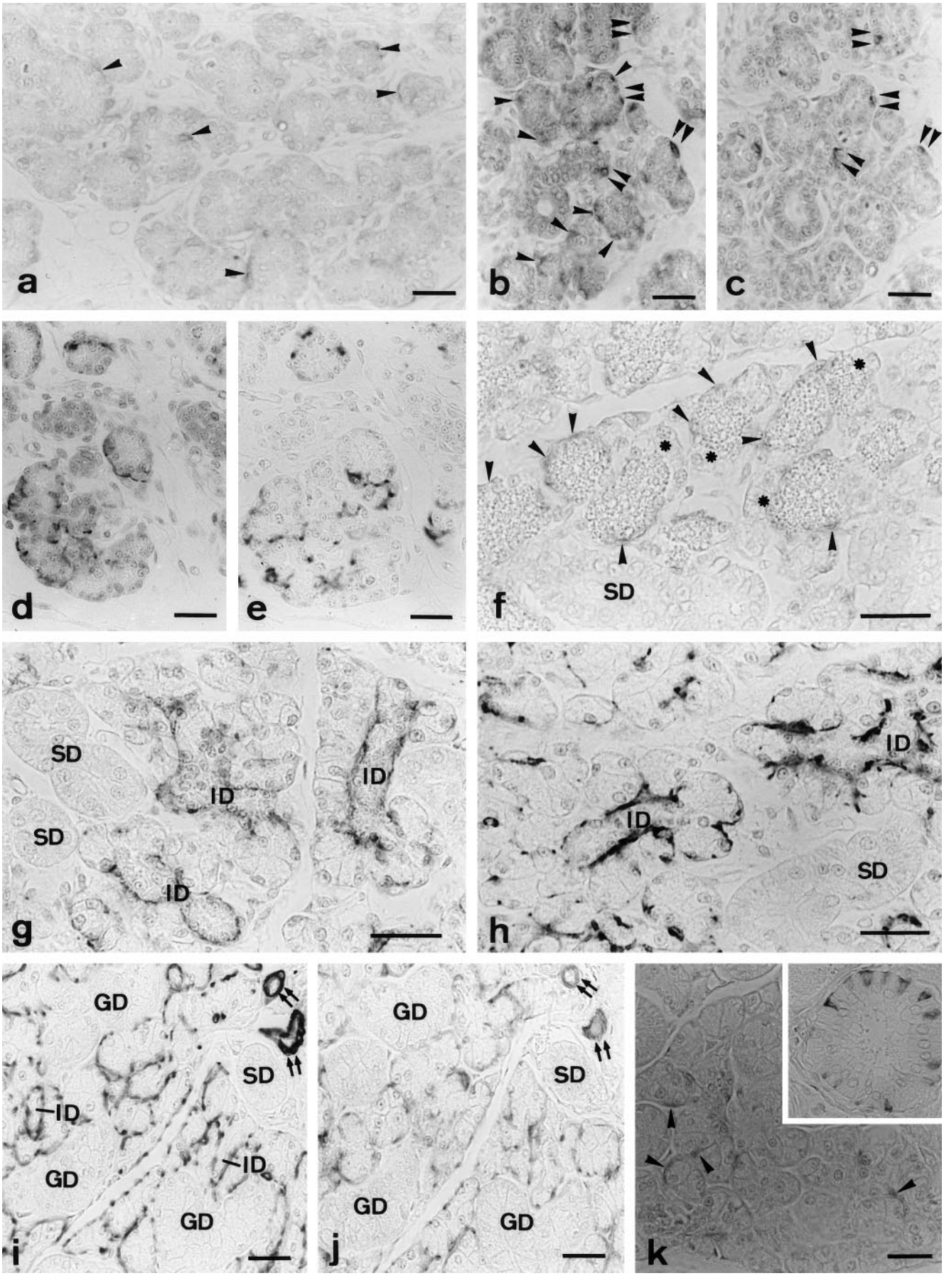
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Table 4 Relative intensity of expression of smooth muscle and epithelial markers by myoepithelial cells in the developing sublingual gland

Age	αSMA[a]		H1-calponin		Keratin 14	
Prenatal	Immature acinus	Small duct	Immature acinus	Small duct	Immature acinus	Small duct
18 Days in utero	0	0	0	0	0	0
19 Days in utero	0	0	±	±	0	0
20 Days in utero	+	+	+	+	0	0
21 Days in utero	++	++	++	++	+	+
Postnatal						
	Acinus	Intercalated duct	Acinus	Intercalated duct	Acinus	Intercalated duct
0 Day	++	++	++	++	+	+
1 Day	++	++	++	++	+	+
3 Days	++	++	++	++	+	+
5 Days	++	++	++	++	+	+
1 Weeks	++	++	++	++	+	+
2 Weeks	++	++	++	++	+	+
3 Weeks	++[b]	++	++[b]	++	+[b]	+
4 Weeks	0	++	0	++	0	+
5 Weeks	++[b]	++	++[b]	++	+[b]	+
Adult	++	++	++	++	+	+

[a] αSMA, α-smooth muscle actin
[b] The number of the positive cells is smaller than in the previous or the following stage

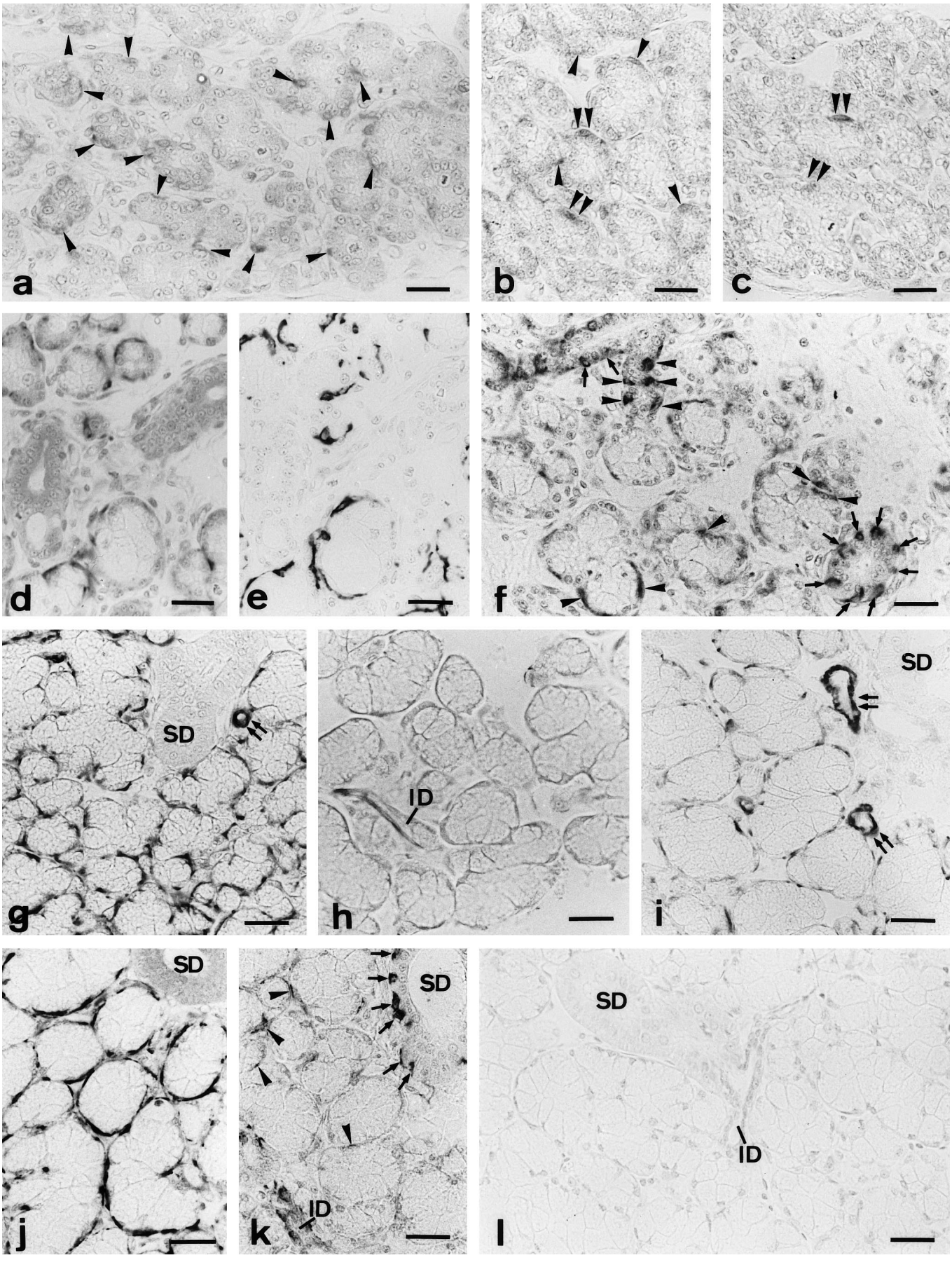
similar to that in the submandibular gland. The MEC differentiation was first detected at 19 days in utero when calponin-positive MECs were sparsely distributed in the outer layer of the developing acini and small ducts adjoining them (Fig. 3a; Table 4). The calponin-positive MECs increased thereafter. At 20 day in utero when the acini had considerably developed and numerous mucous-secreting cells had appeared, some of the MECs acquired αSMA immunoreactivity (Fig. 3b, c; Table 4). The number of the MECs positive for αSMA increased and by birth all the calponin-positive MECs appeared to express αSMA immunoreactivity (Fig. 3d, e). Unlike in the other two major salivary glands, a small number of MECs began to express weak K14 immunoreactivity as early as 21 days in utero (Fig. 3f; Table 4). At birth, the gland had the general over-all appearance of the adult gland with all the three immunohistological markers expressed by the MECs. The mucous acini capped with the serous demilunes and the intercalated ducts had been established already (Table 4). However, the acini were still smaller than in the adult and more loosely arranged within the lobule. After birth, the number of the MECs increased and, at 2 weeks, the acini (Fig. 3g) and the intercalated ducts appeared to be fully invested by the MECs. Between 2 and 4 weeks there was a rapid increase in size of the acini and acinar cells. By 4 weeks, the whole gland appeared compact and was similar to the adult gland. At this stage, the number of the MECs around acini decreased sharply (Fig. 3h) and thereafter increased slowly again (Table 4). The MEC rimming of the acinus at 5 weeks was weaker than in the adult (Fig. 3i; Table 4). In the adult gland, both intercalated ducts and acini were invested by the MECs, which were positive for calponin, αSMA and occasionally K14 (Fig. 3j, k).

Fig. 2a–k Immunoperoxidase histochemistry of rat submandibular glands fixed in methacarn. Calponin (**a**, **b**, **d**, **j**); αSMA (**c**, **e**, **f**–**i**); K14 (**k**). Sections were counterstained with methyl green. **a** 19 Days in utero. MECs are recognized as cells weakly positive for calponin in the outer layer of terminal tubules (*arrowheads*). **b**, **c** 20 Days in utero. Serial sections. Calponin-positive MECs have increased, and some of them express αSMA immunoreactivity (*double arrowheads*). *Arrowheads* indicate calponin-positive, αSMA-negative MECs. **d**, **e** Newborn. Serial sections. All the calponin-positive MECs appear to express αSMA immunoreactivity. **f** 3 Weeks postnatally. Acinar cells and acini are budding out from terminal tubules. MECs are observed in terminal tubules (*arrowheads*) but not in developing acini (*). **g** 4 Weeks. Acini are well developed. Most terminal tubules have developed into intercalated ducts, which are the only structures invested by MECs. **h** 5 Weeks. In addition to intercalated ducts, some parts of acini are rimmed with MECs. **i**–**k** Adult. MECs that enclose both acini and intercalated ducts express immunoreactivity for αSMA (**i**), calponin (**j**) and occasionally K14 (*arrowheads* in **k**). Basal cells of interlobular striated duct also express K14 immunoreactivity (*inset* in **k**). Note αSMA- and calponin-positive vascular walls (*double arrows* in **i** and **j**) (*GD* convoluted granular duct). *Bars* 25 μm (**a**–**e**, **i**–**k** ×320; **f**–**h** ×480)

Alpha-smooth muscle actin, calponin and keratin 14 in cells other than MECs

Antibodies to both αSMA and calponin intensely stained the smooth muscle cells of the vascular walls in addition to the MECs in the salivary glands (Figs. 1f, 2i, j, 3g, i).

K14 immunoreactivity was seen in the cells at the outer or basal layer of the striated and the excretory duct epithelium of all the adult glands (Figs. 2k, 3k). The onset of K14 immunostaining in the duct cells was different among the three glands examined. K14 immunoreactivity in the cells located at the periphery of large ducts was first detected at 20 days in utero in the sublingual gland,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at 21 days in utero in the submandibular gland and at 3 days after birth in the parotid gland. These cells soon developed into the basal cells of the striated and the excretory ducts, and continued to express K14 (Figs. 1e, 3f).

The skin and the oral epithelium in the fetal materials were also positive for K14. In the skin, K14 immunostaining was confined to the basal cells of the epidermis. The staining intensity increased between 19 and 20 days in utero (Fig. 4a, b). In contrast, K14 immunostaining was almost absent in the growing hair follicles (Fig. 4a, b). These features are consistent with those described by Kopan and Fuchs (1989). Though K14 immunostaining in the oral epithelium was seen throughout its thickness, the staining intensity was strongest in the basal layer just before birth (Fig. 4c). This staining pattern was similar to that reported for adult human oral epithelium (Williams et al. 1991; Su et al. 1993).

Localization of β subunit of S-100 protein, vimentin and glial fibrillary acidic protein

Only S-100β immunostaining was seen in the salivary epithelia. In the adult glands, weak S-100β immunostaining was seen in the inner or luminal cells of the striated and the excretory ducts. The convoluted granular duct epithelium in the submandibular gland also exhibited S-100β staining (Fig. 5a). S-100β immunostaining firstly appeared in the cells lining the lumen of the ducts larger than those adjacent to the rudimental secretory structures at 20 days in utero in the submandibular and sublingual glands, and at birth in the parotid gland. These ducts later developed into the striated and the excretory ducts (Fig. 5b). The convoluted granular ducts in the submandibular gland appear from a portion of the striated ducts next to the intercalated ducts after 5 weeks after birth (Jacoby and Leeson 1959).

Fig. 3a–l Immunoperoxidase histochemistry of rat sublingual glands fixed in methacarn. Calponin (**a**, **b**, **d**, **j**); αSMA (**c**, **e**, **g**–**i**); K14 (**f**, **k**). Sections were counterstained with methyl green. **a** 19 Days in utero. Calponin-positive MECs are seen in the outer layer of developing acini and small ducts adjoining them (*arrowheads*). **b**, **c** 20 Days in utero. Serial sections. Acini have developed and mucous-secreting cells have appeared. Some calponin-positive MECs express αSMA immunoreactivity (*double arrowheads*), but others do not (*arrowheads*). **d**, **e** Newborn. The gland is similar to but less compact than adult gland. Almost all the calponin-positive MECs express αSMA immunoreactivity. **f** Newborn. Some MECs express K14 immunoreactivity (*arrowheads*). Basal cells of interlobular striated duct also express K14 immunoreactivity (*arrows*). **g** 2 Weeks postnatally. The number of MECs has increased. Acini appear to be fully invested by them. A vascular wall is positive for αSMA (*double arrow*). **h** 3 Weeks. With a rapid increase in the size of acini and acinar cells, the number of MECs around acini decreases precipitously. **i** 5 Weeks. The number of MECs has increased around acini. Vascular walls are positive (*double arrows*). **j**, **k** Adult. Acini are fully invested by MECs that are positive for calponin (**j**), αSMA and occasionally K14 (*arrowheads* in **k**). Basal cells of interlobular striated duct also express K14 immunoreactivity (*arrows*). **l** Adult. Negative control (normal mouse IgG was substituted for the primary antibody). *Bars* 25 μm (×320)

S-100β and GFAP immunostainings were observed in nerve bundles and their branches in the interlobular and intralobular connective tissues (Fig. 5c). In the fetal material where the entire head and neck region was examined, S-100β immunostaining was also seen in the brain, cartilage, skin, oral mucosa and skeletal muscle, and GFAP staining in the brain. Anti-vimentin antibody stained almost all the above non-epithelial elements. In addition, all the connective tissue elements were stained for vimentin (Fig. 5d). Though round-to-elongated structures positive for the antibodies against S-100β, GFAP and vimentin were sometimes closely associated with the acini and the intercalated ducts, the immunoelectron microscopy confirmed that they were not epithelial (compare Fig. 5d and 5e).

Negative controls

No staining was seen when tissue sections were stained with PBS on normal mouse IgG (Figs. 1k, 3l).

Discussion

We used two smooth muscle-cell-specific antigen markers for identification of MECs in the developing rat salivary glands, namely αSMA and h1-calponin. Among the presently identified six primary isoforms of actin, αSMA has been recognized as a specific marker expressed by the smooth muscle cells (reviewed Rubenstein 1990; Kabsch and Vandekerckhove 1992). During the smooth muscle cell differentiation, the onset of expression of αSMA precedes that of another smooth muscle-specific isoactin γSMA (Ruzicka and Schwartz 1988, Sawtell and Lessard 1989; McHugh et al. 1991). H1-calponin, on the other hand, is considered to be a smooth muscle-cell-specific genomic clone of calponin (Gimona et al. 1990; Strasser et al. 1993; Applegate et al. 1994; Maguchi et al. 1995). This basic protein was first isolated from the chicken gizzard (Takahashi et al. 1986; Takahashi and Nadal-Ginard 1991), and is known to control smooth muscle contraction by binding to actin, tropomyosin and calmodulin (Winder and Walsh 1990; El-Mezgueldi 1996; Winder and Walsh 1996). Immunoreactivities for these markers are known to be highly expressed by MECs of various human and rat glands (Frid et al. 1992; Lazard et al. 1993; Deugnier et al. 1995; Masuda et al. 1996; Fukui et al. 1997).

Before this study, electron microscopy was the most sensitive method for identifying salivary gland MECs during their earliest differentiation (Cutler and Chaudhry 1973a; Redman and Ball 1979; Redman et al. 1980). According to these authors, initial deposition of microfilaments in the MECs was observed at 20 days in utero in the sublingual gland, at 21 days in utero in the submandibular gland and at 1 day after birth in the parotid gland. Previous histochemical studies using actomyosin and alkaline phosphatase activity as probes identified differen-

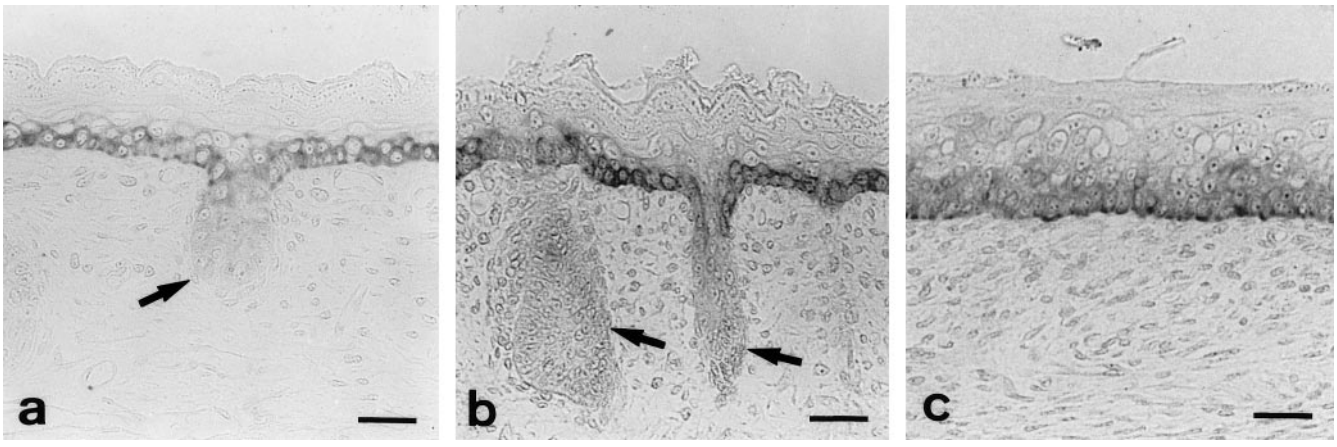

Fig. 4a–c Immunoperoxidase histochemistry of keratin. Tissues were fixed in methacarn. Sections were counterstained with methyl green. **a**, **b** Skin from rat embryos at 19 days in utero (**a**) and 20 days in utero (**b**). K14 imunostaining is confined to basal epidermal cells. The staining is more intense in embryo at 20 days in utero. Note absence of the staining in growing hair follicles (*arrows*). **c** Oral mucosa of embryo at 21 days in utero. K14 immunostaining is strong in the basal epithelial cells. *Bars* 25 μm (×320)

tiating salivary gland MECs only after the onset of electron microscopically detectable deposition of microfilaments (Line and Archer 1972; Redman and Ball 1979; Redman et al. 1980). Therefore, αSMA and calponin used in this study appear to be the known chemical markers that are expressed earliest during MEC differentiation.

Our immunohistochemistry first detected myoepithelial αSMA at 20 days in utero in the sublingual and submandibular glands and at birth in the parotid gland. The immunohistochemically detectable onset of αSMA expression thus coincided with or even preceded slightly the reported appearance of microfilament deposition (Line and Archer 1972; Redman and Ball 1979; Redman et al. 1980). Moreover, calponin expression definitely preceded the αSMA and microfilament deposition. Calponin in the early differentiating MECs has been suggested to play a role in actin polymerization and in making up actin filament bundles (Kake et al. 1995; Kolakowski et al. 1995). Our histochemistry appears to have detected the smooth muscle cell markers, especially calponin, in MECs before the onset of actin polymerization. Later detection by actin immunohistochemistry of parotid gland MECs by Norberg et al. (1996) appears solely due to the lower sensitivity of the primary antibody used (reviewed Mooseker and Cheney 1995).

MECs are believed to be of epithelial origin and have keratin intermediate filaments (Franke et al. 1980). There are at least 20 distinct keratins in the epithelial cells. They are classified into two sequence types (I and II), which are typically coexpressed as specific pairs (reviewed Fuchs and Weber 1994). K14 is a 50 kDa type I keratin and, along with the the type II partner K5, constitutes keratin intermediate filaments in the basal cells of skin epidermis. This pair is also expressed in a number of nonstratified squamous epithelia, in reticular cells and Hassel's corpuscle of thymus, and in parathyroid gland (Fuchs 1996). In human mammary, sweat and salivary glands, the MECs are stained by antibody specific for K14 (Dairkee et al. 1985; Burns et al. 1988; Dardick et al. 1988). In addition, immunostaining for K14 is also seen in the basal cells of the striated and excretory ducts of human salivary glands (Burns et al. 1988; Dardick et al. 1988). The present study demonstrated that both the MECs and the ductal basal epithelia expressed immunoreactivity for K14 in the rat salivary glands as well.

In the developing MECs, K14 immunoreactivity first appeared at 21 days in utero in the sublingual gland, at 5 days after birth in the parotid gland and after 5 weeks postnatally in the submandibular gland. These onsets correspond to the establishment of the acinus-intercalated duct structure in each gland (Jacoby and Leeson 1959; Redman and Sreebny 1971; Redman and Ball 1978). The onset of K14 immunoreactivity expression by the basal epithelia of ducts was also almost simultaneous with the emergence of the duct system (Jadoby and Leeson 1959; Leeson and Booth 1961; Redman and Sreebny 1970; Cutler and Chaudhry 1975; Taga and Sesso 1979).

K14 immunostaining in MECs was weak, and the number of stained cells was small. Therefore, it seems likely that there are two cell types in the salivary gland MECs, i.e., the K14-positive and the K14-negative types. In the rat submandibular and sublingual glands, breaching of the basal lamina was observed in the terminal buds that are composed of undifferentiated cells (Cutler and Chaudhry 1973b; Redman and Ball 1979). Therefore, it may well be that some MECs without K14 is derived from the rudiments seeded by mesenchymal cells at this early developmental stage. We are now conducting a study to examine the expression of other keratin proteins by the MEC.

A great number of studies have shown that "neoplastic MECs" expressed immunoreactivity for S-100 protein, vimentin and GFAP (Caselitz et al. 1981; Hara et al. 1983; Achtstätter et al. 1986, to mention a few). On the other hand, the immunoreactivity for these proteins in normal MECs has recently been questioned (reviewed

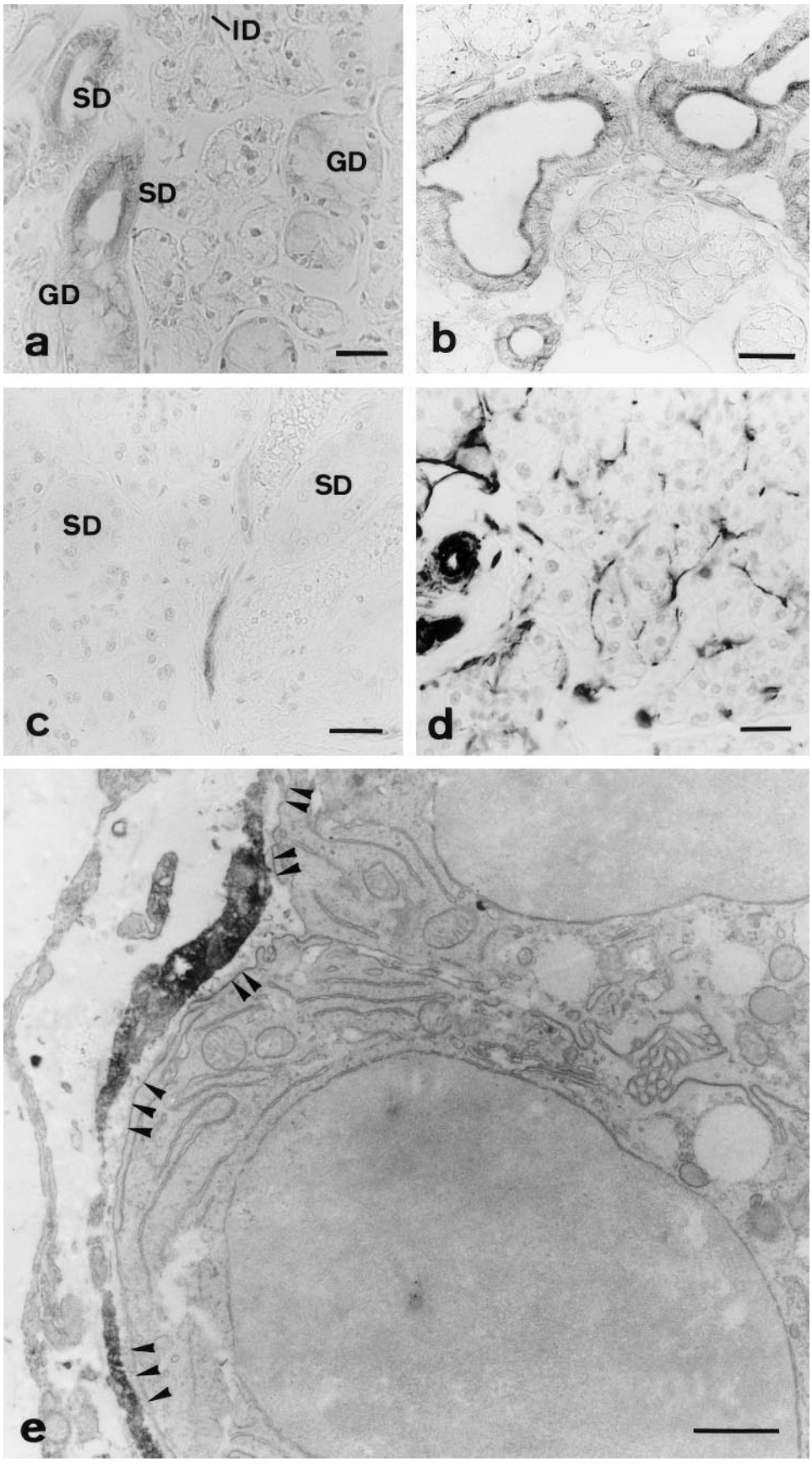

Fig. 5 Light microscopic (**a**–**d**) and electron microscopic (**e**) immunoperoxidase histochemistry of rat salivary glands fixed in 4% paralformaldehyde (**a**, **b**, **e**) and methacarn (**c**, **d**). S-100β (**a**, **b**); GFAP (**c**); vimentin (**d**, **e**). **a** Submandibular gland of adult rat. Luminal cells of striated ducts express S-100β immunostaining. The staining appears to be intense in their luminal cytoplasm. Convoluted granular duct cells also exhibit weak S-100β staining. Intercalated duct is negative. **b** Sublingual gland of 1-day-old rat. Luminal cells of striated ducts express S-100β immunostaining. The staining appears to be confined to the luminal cytoplasm. **c** Submandibular gland of adult rat. Nerve bundles in interlobular connective tissue is positive for GFAP. **d**, **e** Submandibular gland of adult rat (**d**) and parotid gland of 2-week-old rat (**e**). Almost all the connective tissue elements appear to be positive for vimentin (**d**). Although it is not obvious from this staining if some of them are epithelial in origin, by electron microscopy, it is clear that they are not epithelial cells (**e**). Note basal lamina (*arrowheads* in **e**). *Bars* **a**–**d** 25 µm (×320), **e** 1 µm (×12000)

Ellis and Auclair 1996). To our knowledge, Hara et al. (1983) is the only example that unequivocally demonstrated the immunoreactivity for S-100β in the MECs of the submandibular gland. Other investigators demonstrated S-100 immunoreactivity in ducts and/or acinar cells but not in MECs of normal salivary glands (Molin et al. 1984; Hashimoto et al. 1992; Lee et al. 1993; Chisholm and Adi 1995). Vimentin and GFAP have not been demonstrated in normal MECs with any certainty, either. Similarly, the present study did not detect any immunoreactivity for these proteins in MECs in any of the major salivary glands, irrespective of the developmental stage. We observed immunoreactivity for S-100β in the convoluted granular and striated duct cells and their precursor cells. The distribution and time course of development of the ductal S-100β in this study agree well with previous reports for S-100α and/or S-100β (Molin et al. 1984; Lee et al. 1993).

Finally, we should briefly refer to the growth-associated changes in distribution of MECs around the developing acini. To date, topographic changes in MEC distribution has been reported only for the parotid gland. The presently demonstrated MECs in the parotid gland rapidly increased around the terminal buds and proximal ducts perinatally. When the buds differentiated to immature acini at about 1 week, the acinus was thoroughly invested by a monolayer of MECs. The MECs surrounding the developing acini began to decrease at about 3 weeks, that approximately corresponds the time of weaning, and completely disappeared by maturity. Therefore, the time course coincided fairly well with the report by Redman et al. (1980). On the other hand the developmental changes in topographic distribution of MECs has not been investigated in such detail in the sublingual and submandibular glands. In these, the topographic as well as temporal patterns of MEC redistribution were entirely different from each other and from those in the parotid gland. As in the parotid, the sublingual MECs increased to fully invest the developing acinus, and then decreased. Unlike in the parotid, however, the sublingual MECs increased again and the adult acinus regained the full investment by a monolayer of MECs. In the submandibular gland, appearance of MECs around the submandibular acinus was very late compared to the other two salivary glands. At 4 weeks when the acini were well developed, MEC lining has not yet appeared. It was after 4 weeks that MECs first appeared around the acinus, but the adult acini were completely covered by MECs.

What is the functional significance of MEC redistribution in the sublingual and late appearance in the submandibular acini? It is widely accepted in the adult that the hydrodynamic property of saliva is related to the presence and absence of MECs in the salivary gland acini. MECs are known to assist salivary secretion by compressing the underlying parenchyma and/or by providing tensile support for the basis of the acini (Garret and Emmelin 1979; Redman 1994). These functions are probably important for the viscous saliva to pass through narrow and tortuous channels in the sublingual and submandibular glands. The pure serous saliva with a low viscosity does not seem to require this additional MEC support for secretion, hence the absence of acinar MECs in the adult parotid gland. In this context, the acinar MECs in developing parotid gland may not provide a mechanical support for secretion. It may merely represent a transient expression of the immature MEC phenotype that accompanies the initial differentiation of intercalated ducts and acini from their common precursors. Before full maturation, therefore, the acinar MECs disappeared from the parotid gland.

Temporal and morphologic patterns of sublingual gland histogenesis resembled those of the parotid gland; i.e., acinar and duct cell precursors began to differentiate from their common precursors (cord-forming cells) as early as at 19 days in utero. Although acinar cells morphologically similar to mucous cells increased perinatally, a substantial proportion of acinar cells assumed a morphology similar to serous cells (Leeson and Booth 1961) and salivary secretion appeared to be subtle in suckling rats (Schneyer and Hall 1968; Martinez and Camden 1983). Therefore, the increase and decrease of immature sublingual MECs do not appear related to the supportive secretory function assumed by their mature counterparts. Indeed, it is after weaning that the MECs redistributed to the acinus and the demilunes markedly decrease (Leeson and Booth 1961). The re-appeared acinar MECs seem to be of the mature type and support sublingual salivary secretion. As for the submandibular gland, the histogenetic pattern was considerably different from those of the other two glands. The acinar differentiation began much later and was not accompanied by a transient appearance of imamture MECs. However, the emergence of mature acinar MECs was similar in time (after 4 weeks) to that in the sublingual gland.

In summary, smooth muscle markers αSMA and calponin, an epithelial marker K14, and salivary gland tumor markers S-100β, vimentin and GFAP were immunocytochemically examined in the salivery glandular MECs of developing and mature rat salivery glands. None of the examined tumor marker was detected in the MEC irrespective of the developmental stage. Both αSMA and calponin were detected in virtually all the MECs. Immunohistochemically detectable calponin was the earliest expressed marker for the MEC, while the onset of αSMA expression was slightly delayed (by about 1 day). K14 was expressed in a part of the MECs, starting when the acinus-intercalated duct structure was established. The adult MECs were subdivided into two categories on the basis of K14 expression (the K14-positive and -negative types) suggesting a possibility that the some MECs were derived from epithelial cells while others were mesenchymal in origin. Although developing intercalated ducts of all the three major salivary glands acquired MEC lining prenatally, the developmental patterns of acinar MECs varied between the three glands. In the parotid gland, acinar MECs appeared only transiently before weaning and completely disappeared at maturity. In the sublingual gland, they showed a similar transient in-

crease before and decrease during weaning. After weaning, however, the sublingual acinar MECs re-increased to cover the entire acinus at maturity. Submandibular acinar MECs were seen only after the weaning period and fully invested the adult acinus.

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