Int. J. Exp. Path. (1997), **78,** 91–99

Regeneration of myoepithelial cells in rat submandibular glands after yttrium aluminium garnett laser irradiation

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Received for publication 29 August 1996
Accepted for publication 9 January 1997

Summary. The regeneration of myoepithelial cells in rat submandibular salivary gland after partial irradiation with yttrium aluminium garnett (YAG) laser was investigated. The irradiated glands were examined immunohistochemically for actin, histochemically for alkaline phosphatase (ALP), and by transmission electron microscopy (TEM). In control glands, myoepithelial cells were positive for actin and ALP. Electron microscopically, the positive reaction for actin was associated with the myofilaments of myoepithelial cells, and the plasma membrane of myoepithelial cells was positive for ALP. One day after YAG laser irradiation, the irradiated region was necrotic. By 5 days, duct-like structures and epithelial clusters were observed at the interface between the necrotic zone and the remaining undamaged glands; immature acini appeared after 7 days. No reaction in duct-like structures or epithelial clusters to actin or ALP was recognizable by 5 days. However, at 7 days, actin and ALP-positive spindle cells appeared at the periphery of the duct-like structures and immature acini. After 10 days, both actin-positive and ALP-positive cells increased in number. These observations indicate that during regeneration, actin-positive and ALP-positive cells regenerate myoepithelial cells, and it is suggested that this differentiation to myoepithelial cells is closely related to that of luminal to acinar cells. In addition, TEM observations indicate that regenerated myoepithelial cells originated from the basal cells of duct-like structures.

Keywords: regeneration, myoepithelial cell, submandibular gland, laser

Myoepithelial cells are flat cells whose processes embrace acini and ducts of exocrine glands. They are found in mammary (Linzell, 1952), sweat (Hibbs, 1958), lacrimal (Leeson, 1960), prostate (Rowlatt & Franks, 1964) and certain other glands. In salivary glands, myoepithelial cells were first identified by Krause (1865), and many investigators have studied and reviewed their normal structure and development (Young & Lennep, 1977, Garrett & Emmelin, 1979, Redman, 1994). The myoepithelial cells in salivary glands display characteristics of both epithelial and smooth muscle cells (Takahashi, 1958, Tandler, 1965, Harrop, 1968, Leeson & Leeson, 1971) and are thought to play an important role in the histogenesis of several kinds of salivary gland tumours (Batsakis *et al.*, 1983; Dardick *et al.*, 1987; Morinaga *et al.*, 1987). It is clinically

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important to understand regenerative processes in salivary glands. Several investigators have examined the regeneration of ducts and acini in partially injured salivary glands (Milstein, 1950, Hanks & Chaudhry, 1971, Boshell & Pennigton, 1980, Morimura, 1988, Takahashi, 1993, Takahashi & Wakita, 1993, 1994), but few have considered the regeneration of myoepithelial cells. Of these, some (Hanks & Chaudhry, 1971, Takahashi, 1993), but not all (Morimura, 1988), have reported its occurence.

This study was undertaken to determine whether myoepithelial cells are capable of regeneration, and if they are, to clarify the mechanism of regeneration. To achieve this, we examined the regeneration of rat submandibular glands after YAG laser irradiation, using immunohistochemistry for smooth muscle actin, histochemistry for alkaline phosphatase (ALP), and transmission electron microscopy (TEM).

Materials and methods

YAG laser irradiation

Forty-two male Wistar rats, 7–8 weeks old, were used in this experiment. Both submandibular glands were surgically exposed under general anaesthesia with ether and the central portion of the right gland was irradiated with a YAG laser. The conditions of irradiation were: wave length, 1.06 μm; irradiation mode, continuous wave; power, 30 W; time, 1 s; diameter, 2 mm. After YAG laser irradiation the skin wound was sutured. The animals were sacrificed at 1, 3, 5, 7, 10 or 14 days following the irradiation. Animals not exposed to the YAG laser were used as controls.

Immunohistochemistry for actin

After each experimental period the animals were sacrificed by deep inhalation of ether for light microscopy. The submandibular glands were removed immediately and frozen in liquid nitrogen. Frozen sections 5 μm thick were cut with a cryostat, air-dried, and immersed in 4% paraformaldehyde at room temperature for 10 minutes. The sections were rinsed with phosphate-buffered saline (PBS) and incubated with mouse anti-muscle actin monoclonal antibody HHF35 (DAKO). This antibody recognizes smooth muscle α-actin, smooth muscle γ-actin and α-sarcomeric muscle actin, but does not react with non-muscle actin. Biotinylated anti-mouse rabbit polyclonal antibody (DAKO) was applied as the second antibody. The sections were then incubated with streptavidin-biotin horse-radish peroxidase complex (DAKO), and visualized by the 3,3′-diaminobenzidine method. After immunohistochemical staining, the sections were counter-stained with Mayer's haematoxylin.

For immuno-electron microscopy, the animals were perfused with the fixative solution described above for 20 minutes under general anaesthesia with intraperitoneal sodium pentobarbital. The irradiated submandibular glands were dissected out and immersed in the same fixative solution overnight. After fixation, 30-μm cryostat sections were cut and immunostained as above at 4°C. Immunostained sections were post-fixed in 1% osmium tetroxide for 2 hours, dehydrated, and embedded in Epok 812. Ultrathin sections were cut from immunostained sections and examined with a Hitachi H-7000 transmission electron microscope after staining with uranyl acetate for 2 minutes.

Normal mouse serum was substituted for the primary antibody as negative control for light and electron microscopy.

Histochemistry for alkaline phosphatase (ALP)

Cryostat sections were prepared as described for immuno-electron microscopy. The azo-dye method was used to detect alkaline phosphatase activity (Burstone, 1961). Sections 7 μm thick were incubated with 0.1 M Tris-HCl buffer (pH 8.5) containing naphthol AS-MX phosphate as substrate and fast red violet LB salt as diazonium salt for 30 minutes at 37°C and counter-stained with Mayer's haematoxylin.

For TEM, the metal salt method was used (Mayahara *et al.*, 1967). Cryostat 30 μm thick sections were incubated with 28 mM Tris-HCl buffer (pH 8.5) containing β-glycerophosphate as substrate and lead citrate as metal salt for 30 minutes at 37°C, then rinsed with 0.05 M cacodylate buffer. Post-fixation and processing for electron microscopy were performed as described above.

Negative control sections for light and electron microscopy were incubated in medium in the absence of the substrate.

Transmission electron microscopy (TEM)

Specimens for normal TEM were taken from animals sacrificed at 6, 7 or 10 days after YAG laser irradiation. The animals were perfused with 2% paraformaldehyde–1.25% glutaraldehyde buffered at pH 7.4 with 0.05 M sodium cacodylate under pentobarbital general anaesthesia. The irradiated submandibular glands were

immersed in the same solution overnight and rinsed in 0.05 M cacodylate buffer. Post-fixation, dehydration, embedding and ultrathin sectioning were performed in the same way. The ultrathin sections were double-stained with uranyl acetate and lead citrate.

Results

Histological changes during submandibular gland regeneration following YAG laser irradiation have been described previously (Takahashi 1993; Takahashi & Wakita 1993). In summary, one day after irradiation the irradiated area was necrotic and the border between necrotic and viable non-irradiated tissue was well demarcated. Three days after irradiation, duct-like structures and epithelial clusters derived from duct epithelium in adjacent viable tissue (Takahashi, 1993, Takahashi & Wakita, 1993) extended into granulation tissue growing between necrotic and viable tissue. These structures increased in number at 5 days. By 7 days, duct-like structures tended to differentiate and immature acini could be recognized, while epithelial clusters decreased in number. Immature acini were smaller than mature ones and the cells comprising them contained few secretory granules and a round nucleus that occupied a larger proportion of cell cross-section than nuclei of mature acinar cells. Immature and mature acini increased in number after 10 days. By 28 days, no newly formed granular ducts derived from the duct-like structures could be seen.

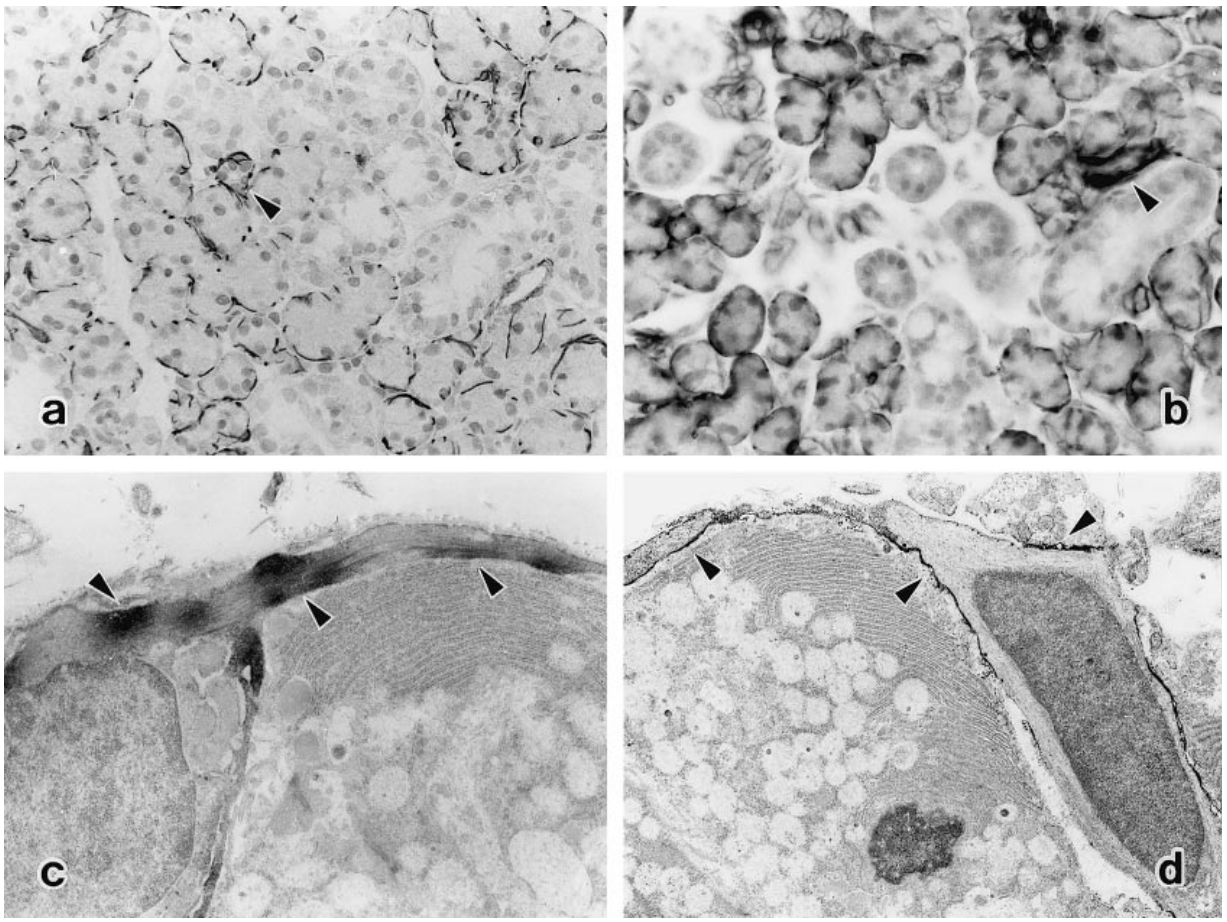
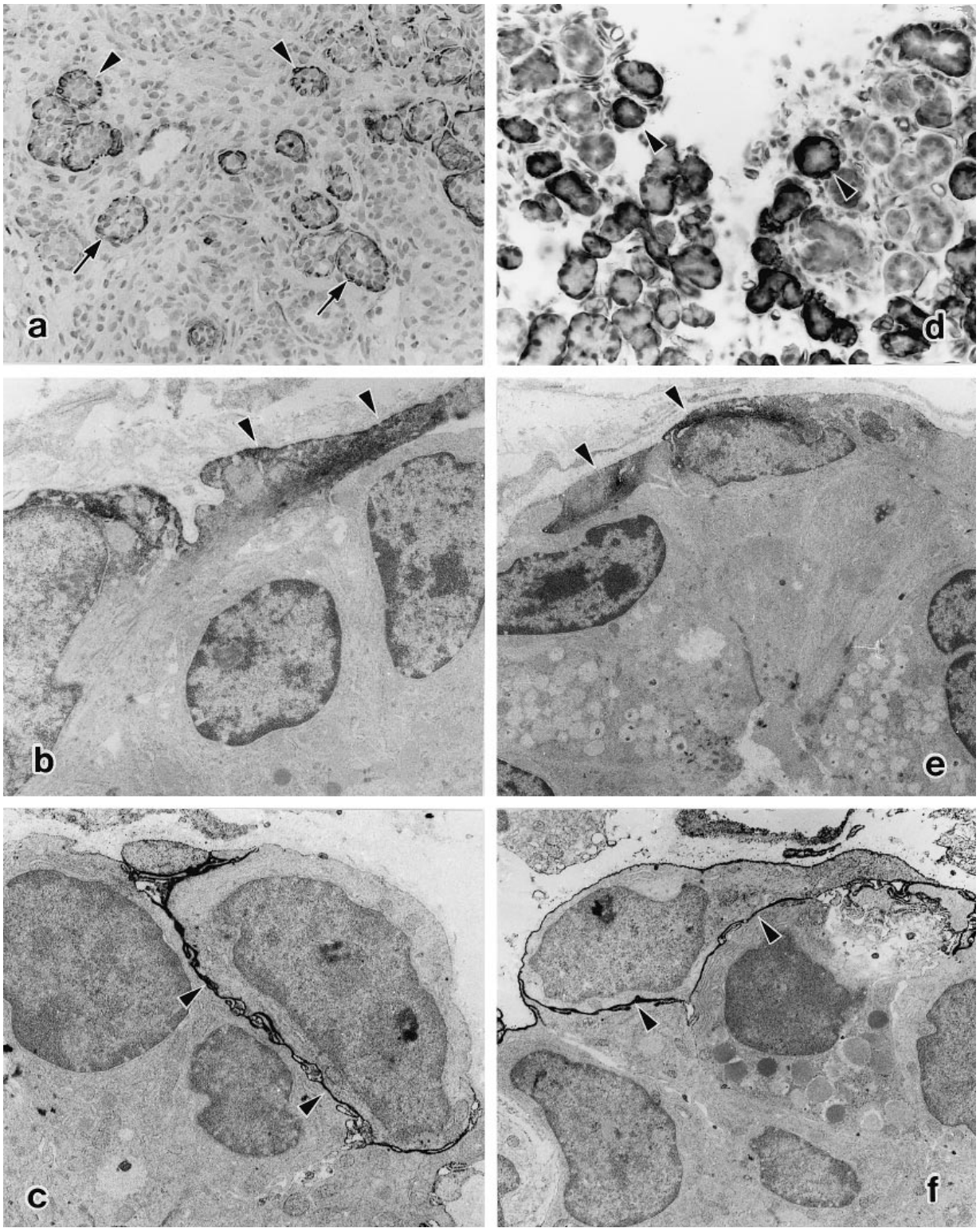


Figure 1. Actin immunohistochemistry and ALP histochemistry of normal submandibular glands. a, Actin-positive cells surround acini and intercalated ducts (arrowhead). There are no actin-positive cells at the periphery of striated or granular ducts. Actin. × 250. b, ALP-positive cells are seen to embrace acini and intercalated ducts (arrowhead), but there are no ALP-positive cells around the striated or granular ducts. ALP. × 250. c, A positive reaction for actin (arrowheads) is shown in a process of a spindle cell external to an acinar cell. Actin. × 8000. d, A spindle cell surrounding an acinar cell shows ALP-positive reaction (arrowheads) along its plasma membrane. ALP. × 5500.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Immunohistochemical and histochemical observations

In control submandibular glands, cells surrounding the acini, and sometimes the intercalated ducts and the blood vessels, showed a positive reaction to anti-actin antibody and ALP. However, there was no reaction in striated or granular ducts (Figure 1a and b). Under the electron microscope, the actin-positive cells appeared flattened and basally located. A positive reaction to the anti-actin antibody was seen in association with cytoplasmic microfilaments (Figure 1c). The ALP-positive cells closely resembled the actin-positive cells; however, the ALP reaction was localized to the plasma membrane (Figure 1d).

One day after irradiation, the cells surrounding acini and some intercalated ducts as well as the smooth muscle of blood vessels in the non-irradiated viable tissue showed a positive reaction to the anti-actin antibody. However, few actin-positive cells were recognized in the necrotic zone. Three days after irradiation, the immunoreaction of remaining viable tissue was unchanged. No immunoreaction was observed in the regenerative duct-like structures or epithelial clusters seen at 3 and 5 days. The reaction to ALP between 1 and 5 days was similar to the results for actin.

Seven days after irradiation, actin-positive and ALP-positive cells were identified at the periphery of some of the duct-like structures and immature acini (Figure 2a). By electron microscopy, cuboidal and low columnar luminal cells of the duct-like structures possessed no or few electron-dense secretory granules, and desmosomes were found between them. The actin-positive reaction was seen in the cytoplasmic processes of spindle cells at the periphery of these cells (Figure 2b). Although the ALP-positive cells were closely similar in appearance to the actin-positive cells, the positive reaction for ALP was localized to cell membrane (Figure 2c). In the period 10–14 days, many immature and mature acini were surrounded by actin-positive and ALP-positive cells (Figure 2d). By electron microscopy, immature acini were seen to be composed of pyramidal cells, some with electron-dense or electron-lucent secretory granules, and flattened cells located peripherally to these cells. The latter showed cytoplasmic positivity for actin (Figure 2e) and membrane positivity for ALP (Figure 2f). Negative control sections for immunohistochemistry and histochemistry showed no reaction.

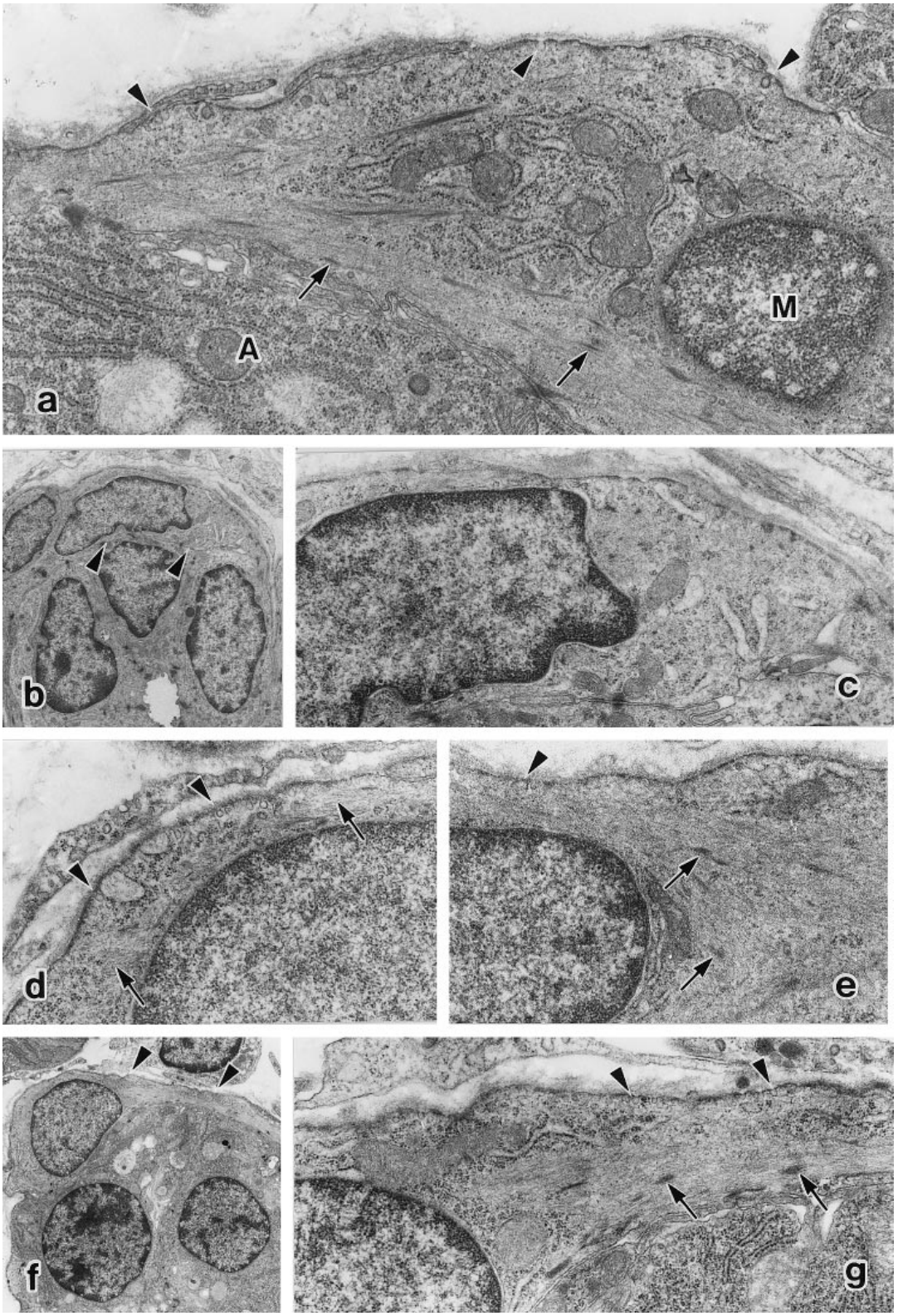
TEM observations

In the control glands, myoepithelial cells of acini were spindle shaped and located between the acinar cells and the basal lamina. They were attached to the acinar cells by desmosomes. Cell organelles were not well developed but some mitochondria, rough endoplasmic reticulum and free-ribosomes were seen adjacent to the nucleus. In other areas of the cytoplasm, especially in cell processes, there were numerous microfilaments running parallel to the long axis of the cell, sometimes associated with dense bodies. There were also caveolae along the basal plasma membrane (Figure 3a).

At 6 and 7 days after irradiation, several types of basal cells could be seen in duct-like structures. The basal cells of the duct-like structures comprising flattened or pyramidal luminal cells were flattened (Figure 3b) and possessed a few mitochondria, rough endoplasmic reticulum, and free ribosomes scattered in the cytoplasm; microfilaments and caveolae were rare. The basal cells were attached to luminal cells by desmosomes (Figure 3c). The basal cells of the duct-like structures, comprising cuboidal or pyramidal luminal cells with round, comparatively large nuclei, contained a few cytoplasmic microfilaments and caveolae in the basal plasma membrane (Figure 3d). In addition, some of basal cells were spindle shaped with extended processes. The latter possessed abundant microfilaments, sometimes with dense bodies, and caveolae were found in the basal plasma membrane (Figure 3e).

Between 7 and 10 days, in addition to the duct-like structures described above there were transitional structures and immature acini. The luminal cells of transitional structures possessed a round nucleus and many cisternae of rough endoplasmic reticulum with an irregular arrangement. In some cells, however, cisternae were arranged in a lamellar fashion and a few secretory

Figure 2. Actin immunohistochemistry and ALP histochemistry of irradiated glands at a–c, 7 days and d–f, 14 days. a, There is an actin-positive reaction in some cells at the outer margin of the duct-like structures (arrows) and immature acini (arrowheads). Actin. ×220. b, A duct-like structure with luminal low columnar cells and basal spindle cells. A positive reaction for actin (arrowheads) is seen in cytoplasm of a spindle cell. Actin. ×8000. c, ALP-positive reaction (arrowheads) is localized to cell membrane of a basal flat cell in a duct-like structure. ALP. ×7600. d, Increased ALP-positive cells surround duct-like structures, immature acini (arrowheads) and mature acini. ALP. ×200. e, An immature acinus comprising inner pyramidal cells and an outer flattened cell. Electron dense or lucent secretory granules are observed in the cytoplasm of pyramidal cells. Cytoplasm of a flattened cell shows positive reaction for actin (arrowheads). Actin. ×5200. f, Positive reactions for ALP are localized to cell membrane of outer cell (arrowheads) of an immature acinus. ALP. ×5500.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granules were seen. The immature acinar cells showed a round nucleus and richer cytoplasm than the luminal cells of the duct-like structures. Though there were several secretory granules and many cisternae of rough endoplasmic reticulum in the cytoplasm of the immature acinar cells, the number of secretory granules was smaller and the arrangement of the rough endoplasmic reticulum was more irregular than in the mature acinar cells (Figure 3f). The basal cells of the transitional structures and immature acini had long processes filled with microfilaments, some dense bodies, and many caveolae at the basal plasma membrane (Figure 3 g).

All basal cells seen during each period were located between the luminal cells and the basement membrane.

Discussion

It is difficult to identify myoepithelial cells by routine histological examination alone (Tandler *et al.*, 1970, Redman & Ball, 1979, Redman *et al.*, 1980) and various methods have been used by investigators to identify these cells in normal and developing glandular tissue. The myoepithelial cells in salivary glands contain muscle actin, which can be detected by immunohistochemistry (Norberg *et al.*, 1992, Lee *et al.*, 1993). These cells also show a positive histochemical reaction for ALP (Redman & Ball, 1979, Redman *et al.*, 1980) shown by TEM to be associated with the cell plasma membrane (Garrett & Harrison, 1970, Garrett & Emmelin, 1979). These two methods are suitable for identification of myoepithelial cells by both light and electron microscopy. Investigations of regeneration of myoepithelial cells have been performed also using immunohistochemistry for actin (Morimura, 1988) and TEM (Hanks & Chaudhry, 1971, Takahashi, 1993) with different conclusions. In the present study, we used three methods: immunohistochemistry with an anti-actin antibody, histochemistry for ALP, as well as TEM to analyse myoepithelial cells regeneration.

In the control submandibular glands, actin-positive and ALP-positive cells were observed at the periphery of acini and intercalated ducts. The positive reaction to actin localized to the microfilaments in the processes of flattened basal cells whose plasma membranes also showed an ALP-positive reaction. These findings agree with the hitherto known characteristics of myoepithelial cells (Garrett & Harrison, 1970, Garrett & Emmelin, 1979, Redman, 1994).

During regeneration, no actin-positive or ALP-positive cells were seen in the duct-like structures and epithelial clusters derived from ducts in adjacent undamaged tissue until 5 days after YAG laser irradiation, suggesting that division and migration of pre-existing myoepithelial cells from the undamaged parenchyma into the newly formed structures did not occur.

At 7 days, actin-positive and ALP-positive cells first appeared at the periphery of the duct-like structures and immature acini, and thereafter increased in number. A positive reaction for actin was identified, by immunoelectron microscopy, in the cytoplasm of spindle or flattened cells at the periphery of the differentiating duct-like structures and immature acini.The same cells also showed a positive reaction for ALP at the cell membrane. These cells were morphologically very similar to normal myoepithelial cells and it is believed they represent myoepithelial cells. The present study thus demonstrates that myoepithelial cells are able to regenerate in partially injured salivary glands. The actin-positive cells in this study resemble the cells around the terminal tubules (Hanks & Chaudhry, 1971) and at the periphery of immature acini (Takahashi, 1993) observed previously by TEM during regeneration of partially damaged rat submandibular glands, suggesting the cells reported in the two papers are also regenerating myoepithelial cells. On the other hand, Morimura (1988), in his immunohistochemical study of rat submandibular gland regeneration after partial resection using an anti-actin antibody, did not identify actin-positive cells around regenerated acini even 56 days after operation, and concluded that myoepithelial cells were not able to regenerate. The reason

Figure 3. TEM of a, the control and b–g, irradiated glands. a, Myoepithelial cell (M) located between acinar cell (A) and the basal lamina. Note abundant microfilaments, sometimes with dense bodies (arrows) in its cytoplasm and caveolae (arrowheads) in the basal plasma membrane. ×20 000. b, Duct-like structure at 6 days. The duct-like structure comprises flattened or pyramidal luminal cells and flattened basal cell (arrowheads). ×4000. c, Higher magnification of the basal cell of the duct-like structure in Figure 3b. Cell organelles are not well developed, and microfilaments and caveolae are rare. ×16 000. d, Basal cell of another duct-like structure at 6 days. A few microfilaments (arrows) in its cytoplasm and caveolae (arrowheads) along the basal plasma membrane are seen. ×15 000. e, Basal cell of the duct-like structure at 7 days. A number of microfilaments sometimes with dense bodies (arrows) in cytoplasm and caveolae (arrowheads) along the basal plasma membrane are observed. ×16 000. f, Immature acinus at 10 days. Immature acinar cells contain several secretory granules. The basal cell extends a very long process (arrowheads). ×3000. g, Higher magnification of the basal cell in Figure 3f. Its process is filled with abundant microfilaments and some dense bodies (arrows). A number of caveolae (arrowheads) are seen along the basal plasma membrane. ×16 000.

for this difference is unknown but may be due to variation in the specificity or sensitivity of the anti-actin antibodies used in his and our studies.

TEM was used to determine the origin of the regenerated myoepithelial cells. Ultrastructural features of normal myoepithelial cells in salivary glands may be summarized as follows: they are spindle-shaped and located between the acinar cell and basement membrane; they contain abundant myofilaments (microfilaments), electron-dense bodies and caveolae at the basal plasma membrane (Tandler, 1965, Harrop, 1968, Cutler & Chaudhry, 1973, Redman & Ball, 1979, Garrett & Emmelin, 1979, Redman *et al*., 1980, Redman, 1994). During regeneration in the present study, the basal cells of duct-like structures at 6 and 7 days were located between the luminal cell and basal lamina, but they did not possess other characteristics of myoepithelial cells. During the differentiation of luminal cells of duct-like structures, the basal cells gradually acquired microfilaments and other characteristics of myoepithelial cells, while the basal cells of the transitional structures and immature acini came to assume the full features of myoepithelial cells. These findings suggest that regenerating myoepithelial cells are derived from the basal cells of duct-like structures, and that their differentiation to myoepithelial cells is closely associated with the differentiation of luminal cells to acinar cells. It is further suggested that regenerating myoepithelial cells are of epithelial origin as the basal cells are always located inside the basal lamina.

During the development of salivary glands, myoepithelial cells are initially identified in the terminal buds and terminal tubules by their basal position and elongated shape. Next, the myofilaments and plasmalemmal caveolae appear (Cutler & Chaudhry, 1973, Redman & Ball, 1979, Redman *et al*., 1980, Redman, 1994). The regeneration of the myoepithelial cells demonstrated here therefore is very similar to the developmental process.

In conclusion, this study has shown that during submandibular gland regeneration, the actin and ALP positive cells at the periphery of duct-like structures and immature acini are newly regenerated myoepithelial cells. The TEM observations suggest that the regenerating myoepithelial cells originate from the basal cells of duct-like structures, and that the differentiation to myoepithelial cells is closely related to that of luminal cells to acinar cells.

Acknowledgements

The authors gratefully acknowledge the assistance of Dr Neal I. Walker and Mr Clay Winterford of the Department of Pathology, University of Queensland Medical School, Australia, with the manuscript and figures, respectively.

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