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The pathobiology of salivary gland

I. Growth and development of rat submandibular gland organoids cultured in a collagen gel matrix

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Summary. Fragments of rat submandibular gland (organoids) which maintained the topological organization of the parent tissue were cultured in a three-dimensional collagen gel matrix for up to 30 days. At 48 h, vigorous peripheral outgrowth had occurred around each organoid. This was accompanied by central necrosis and the bridging of adjacent organoids. By day 5, large cyst-like spaces occupied the centre of many organoids. Bromodeoxyuridine labelling indicated that a considerable proportion of the lining cells were proliferating. Organoid growth peaked at between 5 and 10 days. Thereafter, the number of viable colonies and proliferating cells declined. Addition of isoproterenol after 24 h culture resulted in marked morphological alterations, with earlier and more prolific outgrowth and a greater tendency for organoids to flatten and grow out over the surface of the gel with squamous differentiation. Ultrastructurally, nuclear and cytoplasmic features of isoproterenol-treated and untreated cultures were similar. The secretory granules and extensive rough endoplasmic reticulum of terminal tubule cells, evident in organoids immediately after isolation, were infrequent after 24 h and absent by 48 h. Similar alterations occurred in the few acinar cells, so by 5 days the cultures were composed entirely of a uniform population of primitive, dedifferentiated cells. Further uses of this culture systems will include the study of diseases and disorders of the salivary glands as well as normal growth and differentiation pathways.

Key words: Submandibular gland – Tissue culture – Electron microscopy – Proliferating cells

Introduction

In recent years, there has been an upsurge in interest in the salivary glands and their pathology (Dardick and van Nostrand 1987; Martinez-Madrigal and Micheau 1989). However, research has been hampered by the lack of a reliable in vitro system for investigating the growth and differentiation of salivary gland tissue under normal and experimental conditions. Explants of human submandibular and minor salivary glands can be successfully grown for a period of time, but the outgrowth from the explants is monolayered and predominantly of duct-type epithelial cells (Sens et al. 1985; Kurth et al. 1989), so that tissue organization is not comparable to that seen in vivo. Suspensions of dissociated rat submandibular gland cells, which are more representative of the parent tissue, have been prepared and studied in short-term culture (Kanaamura and Barka 1975). Following dissociation, the cells retained their in vivo structural characteristics. However, by 36 h the amount of secretory material in acinar cells was reduced and the β-adrenergic agonist isoproterenol, a potent secretagogue and growth stimulant of submandibular glands, failed to induce discharge of secretory material or incorporation of tritiated-thymidine into cellular DNA (Kanamura and Barka 1975). This suggests that in short-term culture, suspensions of submandibular gland cells rapidly lose both their functional and proliferative capacity. The object of our study was to develop an in vitro system for culturing salivary glads in which the structural relationships between the different cell types present in the gland, and therefore the interaction between them, might be maintained. A number of different culture techniques have been devised where three-dimensional tissue organization is retained or develops in vitro. Mini-organs of thyroid tissue have been grown in hydrophobic plates that prevent attachment and preserve the structural characteristics of intact thyroid tissue (Bauer and Herzog 1988). The differentiation of retinoblastoma cell lines has been achieved using Gelfoam matrices (Herman et al. 1989) and transformed human salivary glad cells have been grown in a gelatin sponge matrix (Shirasuna et al. 1981). Explants of mouse embryonic submandibular gland have been used to study the interaction of epithelium and adjacent mesenchyme in ordering gland shape (Nakanishi and Ishii 1989), while similar culture techniques followed stages in fetal mouse palatal development (Shiota et al. 1990).

Collagen gel matrices have been used successfully as

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a substrate in histiotypic and organ cultures of normal and neoplastic tissues such a breast, gall bladder, pancreas and thyroid (Yang et al. 1979; Burwen and Pitelka 1980; Ormerod and Rudland 1982; Foster et al. 1983; Chen et al. 1985; Kawamura et al. 1989; Toda and Sugihara 1990). The maintenance of ductal structure and the development of new, small ductules when human breast tissue fragments were grown in rat-tail collagen gel matrices (Foster et al. 1983) suggested that similar results might be obtained with salivary gland tissue. Using this method, we have successfully grown intact fragments of dissociated rat submandibular gland (termed organoids) and have examined their development using light and electron microscopic techniques.

Materials and methods

Eight-day-old Wistar rat pups were used. At this age, the submandibular gland is still in an active growth phase (Chang 1974; Klein 1982), but fully differentiated myoepithelial cells as well as striated duct and secretory cells are present (Cutler and Chaudhry 1973, 1975). Six animals were used in each experiment. In order to compare the submandibular gland in vivo with the isolated tissue used for preparation of the organoids, one rat was injected with the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU), 30 mg/kg body weight intraperitoneally, 1 h prior to sacrifice. The submandibular glands from this animal were removed, cleaned of fat and fascia, portions fixed for light microscopy and some cut into 1-mm cubes which were fixed for electron microscopy. Submandibular glands from the remaining five animals were used for preparation of the organoids.

Organoids were prepared using a combination of mechanical and enzymatic tissue dissociation. The glands were removed aseptically, minced finely in a little culture medium and then digested according to a modification of the method of Barka and Van der Noen (1975). The tissue was incubated at 37° C for 1 h in minimum essential medium with Earle's salts and 25 mM Hepes buffer (MEM) culture medium containing collagenase (200 μg/ml), hyaluronidase (2 μg/ml) and DNAase (5 μg/ml); these and other additives were obtained from Sigma Chemical Co., St. Louis, MO. After incubation, the tissue fragments were disaggregated by gentle pipetting. The tissue pellet was resuspended in MEM and passed through a sieve with a 100-μm mesh. Any large tissue fragments remaining on the mesh were pressed through using a cell scraper. Organoids prepared in this way contained clusters of duct and terminal tubules less than 100 μm in diameter. The organoids were then washed twice in MEM and resuspended in 5 ml collagen gel (Vitrogen 100, ICN Flow, Mississauga, Canada). The gel was prepared according to the manufacturers instructions with the addition of the growth factors insulin (5 μg/ml), hydrocortisone (5 μg/ml), epidermal growth factor (50 ng/ml), and cholera toxin (25 ng/ml). The organoids were seeded in aliquots of 0.35 ml into 12-mm Millicell-CM well inserts (Millipore, Bedford, MA) in 6-well culture plates (ICN Flow, Mississauga, Canada), four inserts to each well, and incubated at 37° C in an atmosphere of 5% CO_2. After gelation had occurred, 4 ml growth medium [MEM culture medium as above plus penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml) and 10% serum] was added to each well of the culture plate. Growth medium (150 μl) was also added to each well insert so that the gels were perfused from above and below. Other workers have suggested that the inclusion of non-physiological components such as fetal bovine serum in experimental protocols may invalidate comparisons of in vitro and in vivo studies (Reisser et al. 1989). Pooled adult rat serum was therefore used throughout the study, initial experiments having indicated that medium supplemented with allogenic serum enhanced the growth of the organoids compared to the medium supplemented with fetal bovine serum. After feeding, cultures were reincubated, and thereafter, were fed every 5 days unless otherwise indicated.

In some studies, growth medium was removed after 24 h and replaced with growth medium containing isoproterenol (200 μg/ml) or fresh growth medium alone for control cultures. The cultures were incubated with isoproterenol for 24 h, then washed with warm, sterile phosphate-buffered saline and finally fed with growth medium and reincubated. Control cultures were similarly treated.

Individual gels were fixed at various time intervals in methanol/acetic acid (90:10 v/v). After gels had been embedded in paraffin, 5-μm sections were stained with haematoxylin and eosin.

Cellular proliferation was monitored in cultures by exposing gels to BrdU at a concentration of 3 μg/ml in MEM culture medium plus 10% rat serum for 1 h prior to fixation. Incorporation of BrdU into replicating DNA was exposed using a monoclonal antibody to BrdU (Cell Proliferation kit RPN.20, Amersham). Bound antibody was detected using an indirect immunoperoxidase method and 3,3′-diaminobenzidine in the presence of cobalt and nickel salts.

Methanol/acetic acid-fixed and paraffin-embedded tissue from adult and 8-day-old rat submandibular gland was stained using an indirect immunoperoxidase technique and a monoclonal antibody to muscle-specific actin (HHF35, 1:4000; Enzo Biochem, New York, N.Y.).

At different time intervals, gels were processed for electron microscopy. Samples were fixed in 2% gluteraldehyde in 0.1 M phosphate-buffered saline, post-fixed in 1% osmium tetroxide in the same buffer, dehydrated through alcohols and embedded in epon/araldite resin. Semithin sections were stained with methylene blue to select appropriate organoids for study. Ultrathin sections were stained with uranyl acetate and lead citrate prior to examination and photography on a Philips EM400 electron microscope at 60 kV.

Results

Characterization of in situ gland

At 8 days of age, rat submandibular gland was still immature and consisted of striated and intercalated ducts leading to terminal tubule "end buds" (Fig. 1A). Ultrastructurally, terminal tubule cells were richly endowed with rough endoplasmic reticulum and apically contained a modest number of round, densely staining secretory granules (Fig. 1B, C). A few acinar cells with more numerous, compactly organized electron-lucent granules, some with internal, punctate to linear, darker staining patterns were evident between the terminal tubule cells. Intercalated duct cells were smaller in comparison to the terminal tubule cells and were devoid of secretory granules (Fig. 1B). Both the intercalated ducts and terminal tubules were bordered by myoepithelial cells (Fig. 1B, C). Immunostaining for muscle-specific actin (Fig. 2A) clearly highlighted these cells and their cytoplasmic processes, and was comparable to the staining seen in the submandibular gland of the adult rat (Fig. 2B). That the principal cell-types present in the submandibular gland of the 8-day rat were actively proliferating was clear from BrdU labelling (Fig. 2C).

Isolated organoids

Toluidine-blue-stained epoxy sections of isolated organoids, after 1 h of culture in the collagen gel matrix, demonstrated that the majority of organoids consisted of terminal tubule/intercalated duct segments (Fig. 3A, B);

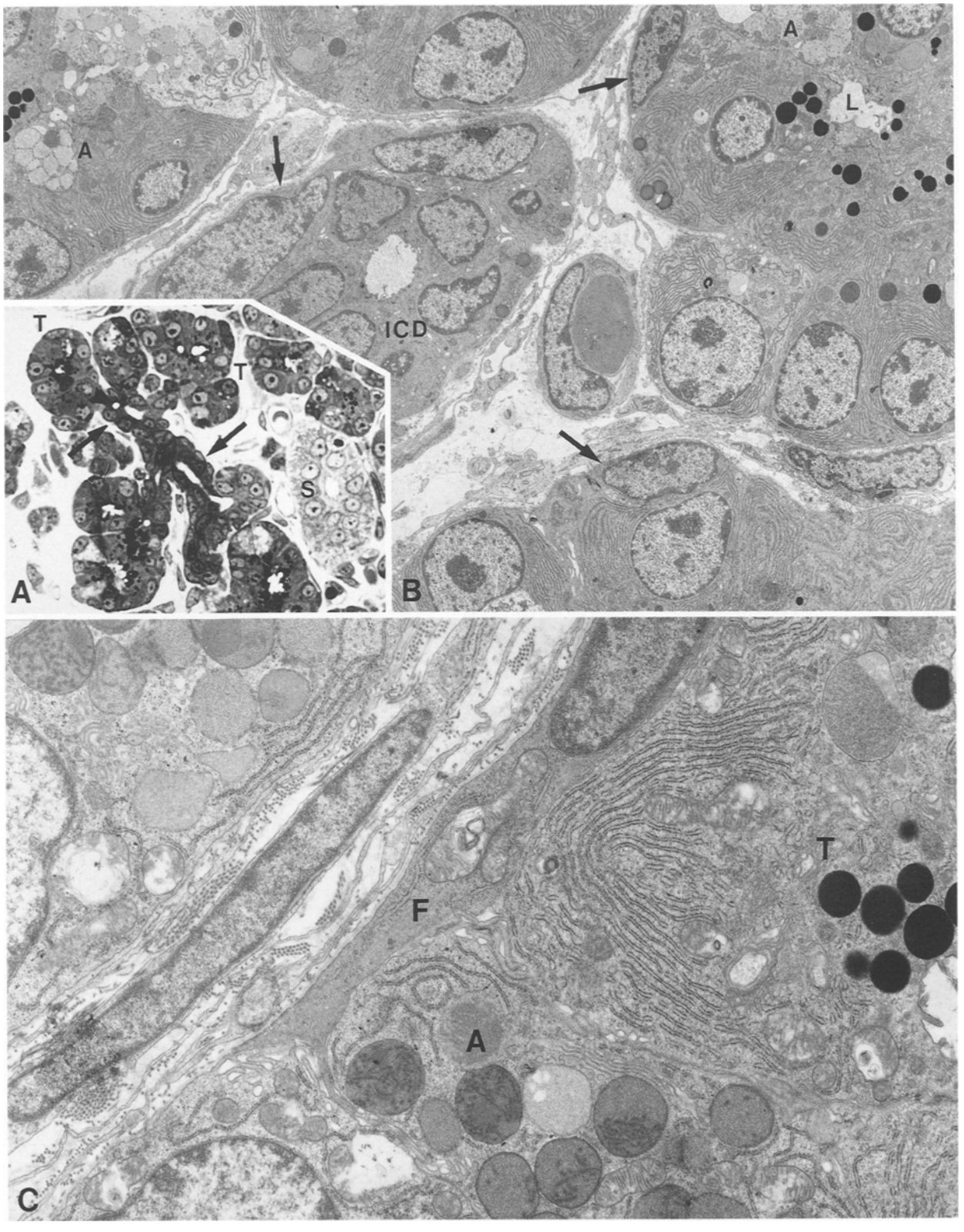

Fig. 1 A–C. Rat submandibular gland 8 days after birth. **A** Terminal tubules (*T*) or "end buds", composed of cells with darkly staining apical granules, arise from segments of intercalated ducts (*arrows*). **B** Terminal tubules are mainly composed of cells with densely staining, apical secretory granules, that form small central lumens (*L*), and some basally situated myoepithelial cells (*arrows*). A few acinar cells (*A*) with paler staining cytoplasmic granules are present. Intercalated ducts (*ICD*) are composed of luminal cells and basal oriented myoepithelial cells. **C** A rough endoplasmic reticulum-rich terminal tubule cell (*T*) is adjacent to an acinar cell (*A*) with paler staining pleomorphic appearing granules. The process of a flattened myoepithelial cell is rich in filaments (*F*). **A** Toluidine blue-stained epon section, ×500; **B, C** uranyl acetate and lead citrate (UA&LC), ×3300 and ×12000, respectively

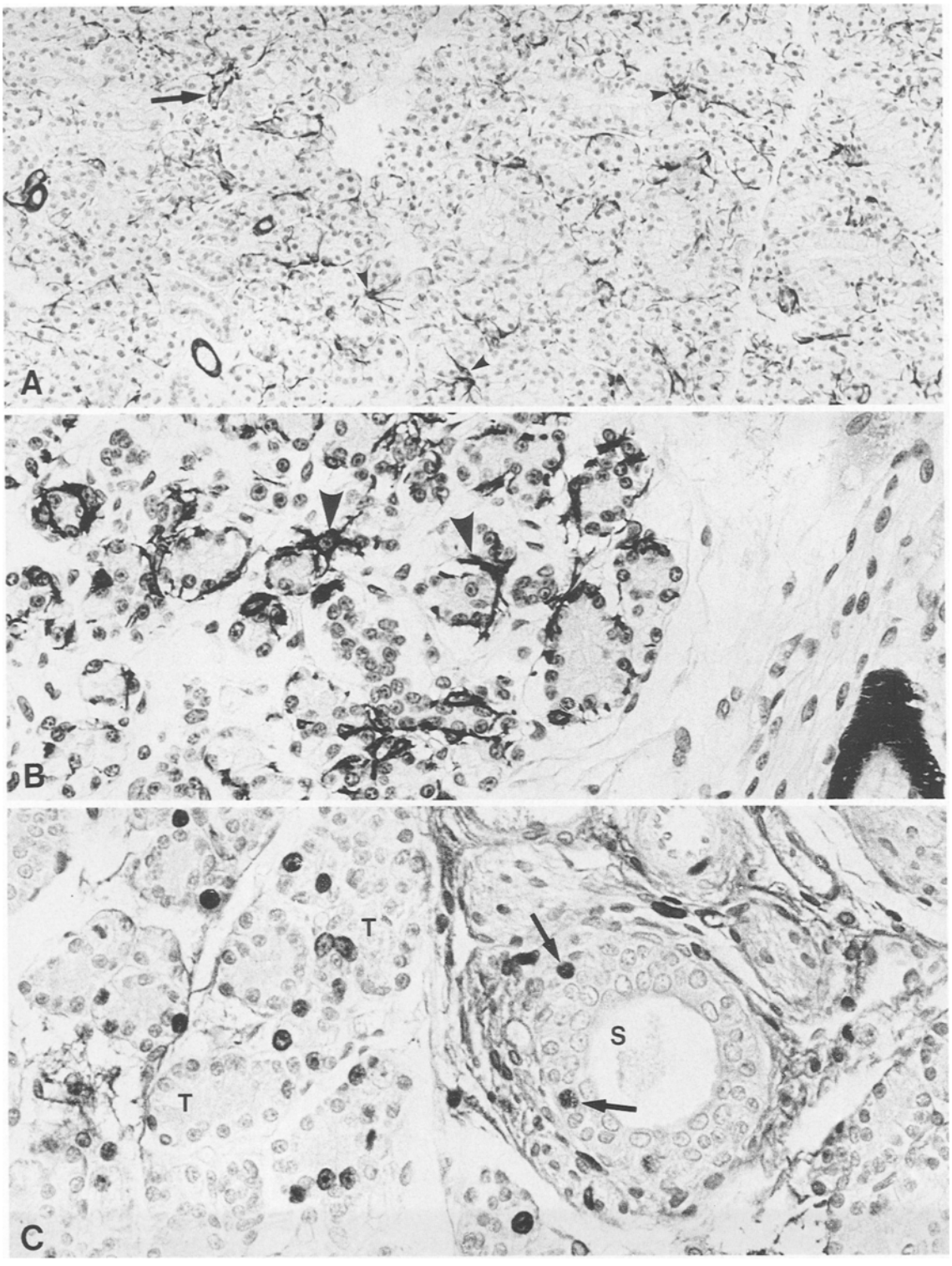

Fig. 2. A Adult rat submandibular gland immunostained for muscle-specific actin has many acini and intercalated ducts (*arrow*) with peripherally oriented myoepithelial cells. Note the many fine cellular processes associated with myoepithelial cells (*arrowheads*). **B** At 8 days of age, submandibular gland stained for muscle-specific actin has identifiable myoepithelial cells some with well-developed cellular processes (*arrowheads*). **C** Bromodeoxyuridine (BrdU) has been incorporated in many of the nuclei of terminal tubule cells (*T*) of 8-day-old rat submandibular gland, but striated ducts (*S*) are also labelled (*arrows*). Immunoperoxidase. **A**, ×200; **B, C** ×500

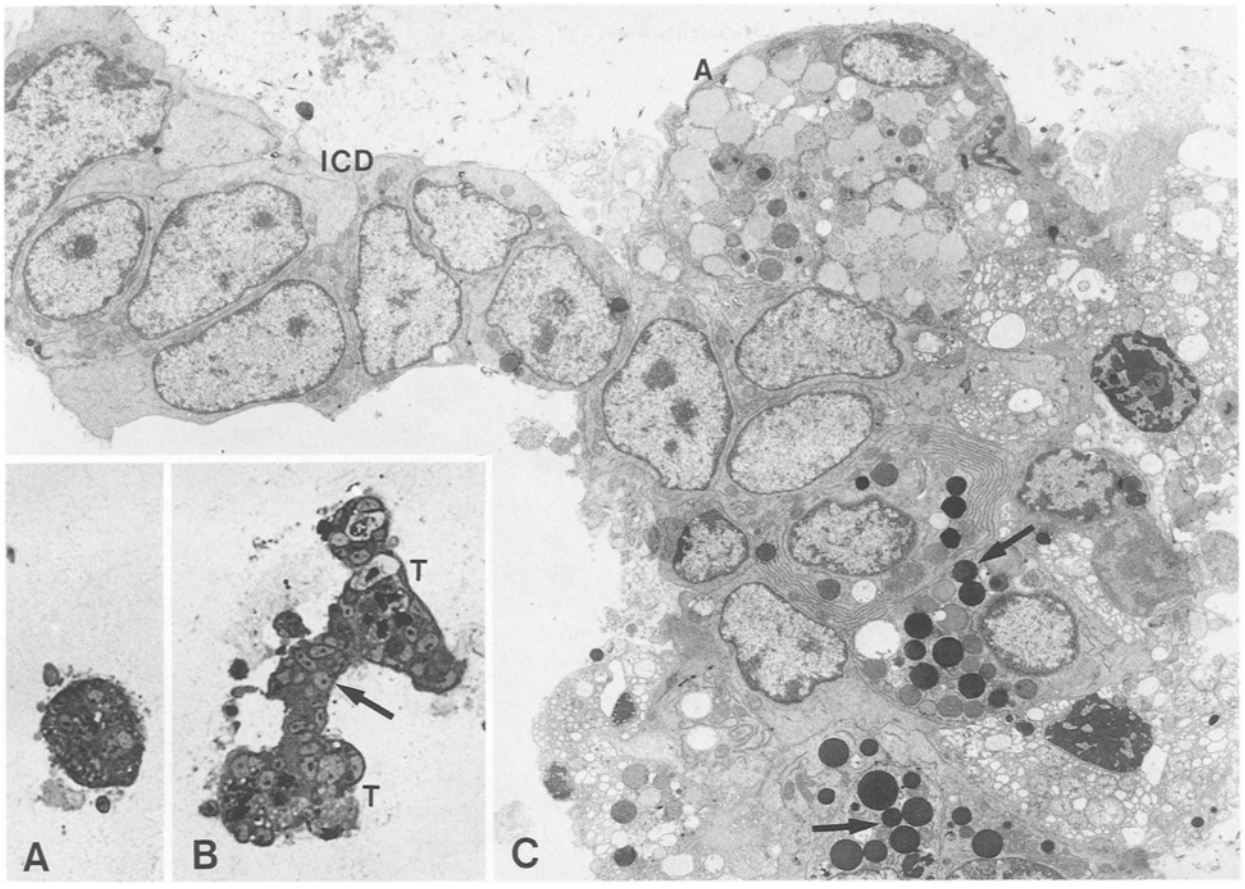

Fig. 3A–C. Isolated organoids of 8-day-old rat submandibular gland. **A** Gland fragments consist of portions of terminal tubules. **B** Other organoids composed of terminal tubules (*T*) have attached segments of intercalated ducts (*arrow*). **C** The combined intercalated duct (*ICD*) and terminal tubule seen in **B** consists of intact terminal tubule cells with electron-dense granules (*arrows*) and acinar cells (*A*) containing zymogen granules. **A, B** Toluidine blue-stained epon sections, ×350; **C** UA & LC, ×3300

a feature confirmed ultrastructurally (Fig. 3C). Acinar cells, where present, and terminal tubule cells still retained their secretory granules. Myoepithelial cells were identified by the presence of myofilaments at the ultrastructural level. Although there was evidence of damage at the periphery of the organoids (Fig. 3C), cell viability and non-disrupted tissue organization was confirmed ultrastructurally. The epithelial cells showed intact organelles and the presence of tight junctions and desmosomes, as well as glandular lumens with microvilli.

Cell cultures

Phase-contrast microscopy. Under phase-contrast microscopy, the isolated fragments of rat submandibular gland had an irregular, somewhat bulbous appearance and some bore a projecting segment of intercalated duct (Fig. 4A). Changes in the morphology of the organoids were evident 48 h after the initiation of culture (Fig. 4B). The organoids grew within the gel matrix as spheroidal colonies of variable size, slightly larger than the original isolates (Fig. 4A), with fairly regular margins but with outgrowths of a few peripheral cells (Fig. 4B).

By 5 days of culture, the organoids had rounded up and appeared denser and more compact (Fig. 4C). Peripheral cell outgrowth, now more apparent, was preferentially directed towards adjacent organoids, and bridging of organoids was common. Addition of isoproterenol to the cultures, 4 days previously, had a marked effect on organoid morphology and size by day 5 (Fig. 4D). The original margins were now obscured by a prolific outgrowth of blunt or pointed, duct-like processes that eventually fused with adjacent organoids (Fig. 4D).

By 10 days, both untreated and isoproterenol-treated cultures had become dense and compacted so that it was no longer possible to assess growth of the cultures in situ.

Light microscopy. In histological sections of collagen gels examined after 24 h in culture, organoids showed little peripheral outgrowth, some central necrosis, and only the occasional BrdU-labelled nucleus. By 48 h, vigorous peripheral outgrowth had occurred along with the development of new intercellular spaces between epithelial cells (Fig. 5A); approximately 5% of the cells labelled positive for BrdU and were randomly distributed throughout the organoids. Organoids which had earlier been exposed to isoproterenol revealed little difference in morphology at 48 h (Fig. 5B), but contained less than

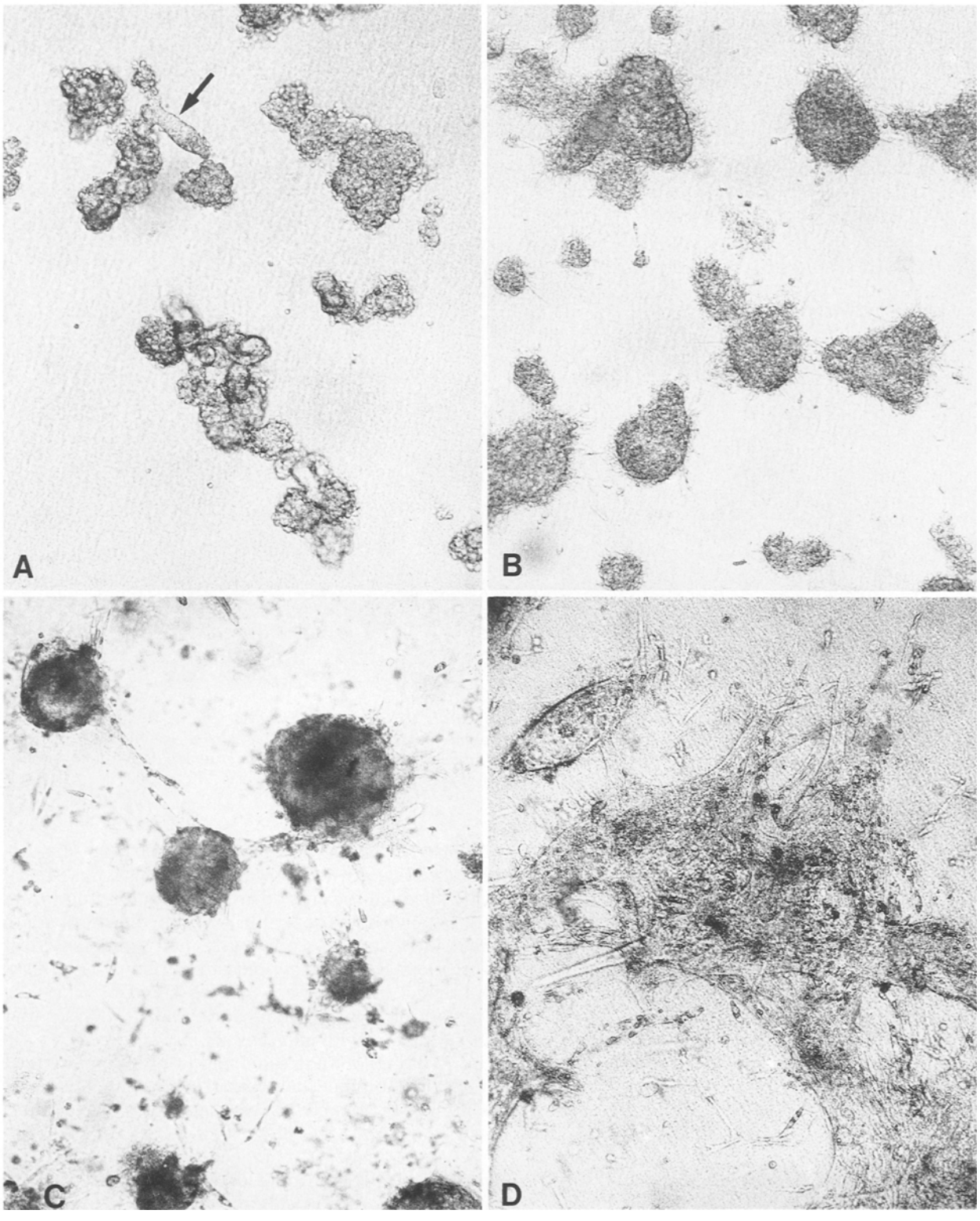

Fig. 4A–D. Phase contrast microscopy of 8-day-old rat submandibular gland organoids in collagen gel culture. **A** Shortly after embedding in the collagen gel, isolated organoids consist of lobulated appearing terminal tubules, some with attached segments of intercalated ducts (*arrows*). **B** After 48 h in culture (no drug), the organoids are somewhat larger, less lobulated and have fine cellular processes projecting from the surface. **C** At 5 days of culture (no drug), the organoids have a dense, homogeneous appearing central zone (probably corresponding to the cystic luminal space seen in Fig. 6C) and thicker, focal cellular projections mainly oriented towards adjacent organoids. **D** Cultures at day 5 after exposure to isorproterenol reveal considerable enlargement of the organoids with many relatively thick triangular-shaped projections. Some processes have fused with adjacent organoids. **A** $\times 380$; **B–D** $\times 300$

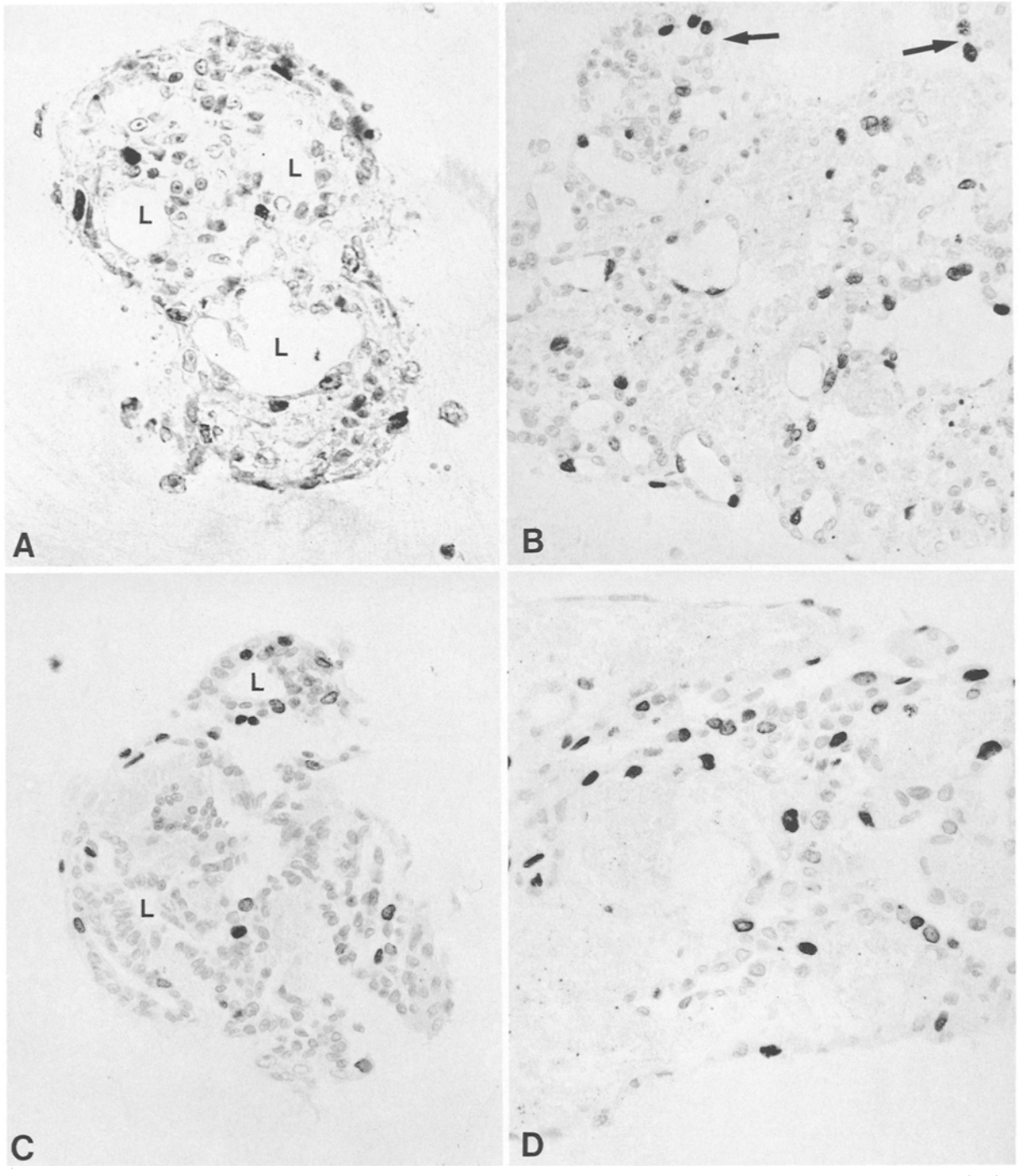

Fig. 5A–D. Histological sections of 8-day-old rat submandibular gland cultured in collagen gel matrix and exposed to BrdU prior to fixation. **A** At 48 h of culture (no isoproterenol), multiple lumens (*L*) and a moderate number of labelled nuclei (*black*) are present. **B** Organoids at 48 h of culture after exposure to isoproterenol have scattered labelled nuclei and some bulbous cellular outgrowths (*arrows*). **C** At day 5 of culture (no isoproterenol), organoids still have a cellular interior, a well-organized epithelium, persisting lumens (*L*) and continued labelling of nuclei. **D** By day 10 of culture (no isoproterenol), multiple lumens are still present, the central zones contains well-organized cells, and BrdU-labelled nuclei are seen both peripherally and centrally. Immunoperoxidase using anti-BrdU antibody with nuclear fast red counterstain. **A–D** $\times 350$

2% BrdU-positive cells compared to control cultures (Fig. 5A).

By day 5, large cyst-like spaces had developed in the centre of many of the organoids (Fig. 5C). The cells contained in these were actively proliferating and between 50 and 70% of the nuclei incorporated BrdU (Fig. 5C). Isoproterenol-treated organoids developed the same cystic spaces, but BrdU-labelled cells were limited to 10–15% of the population. By day 10 of culture in the gel matrix (Fig. 5D), central regions of the organoids again contained many cells, up to 25% of which continued to incorporate BrdU, and multiple lumens

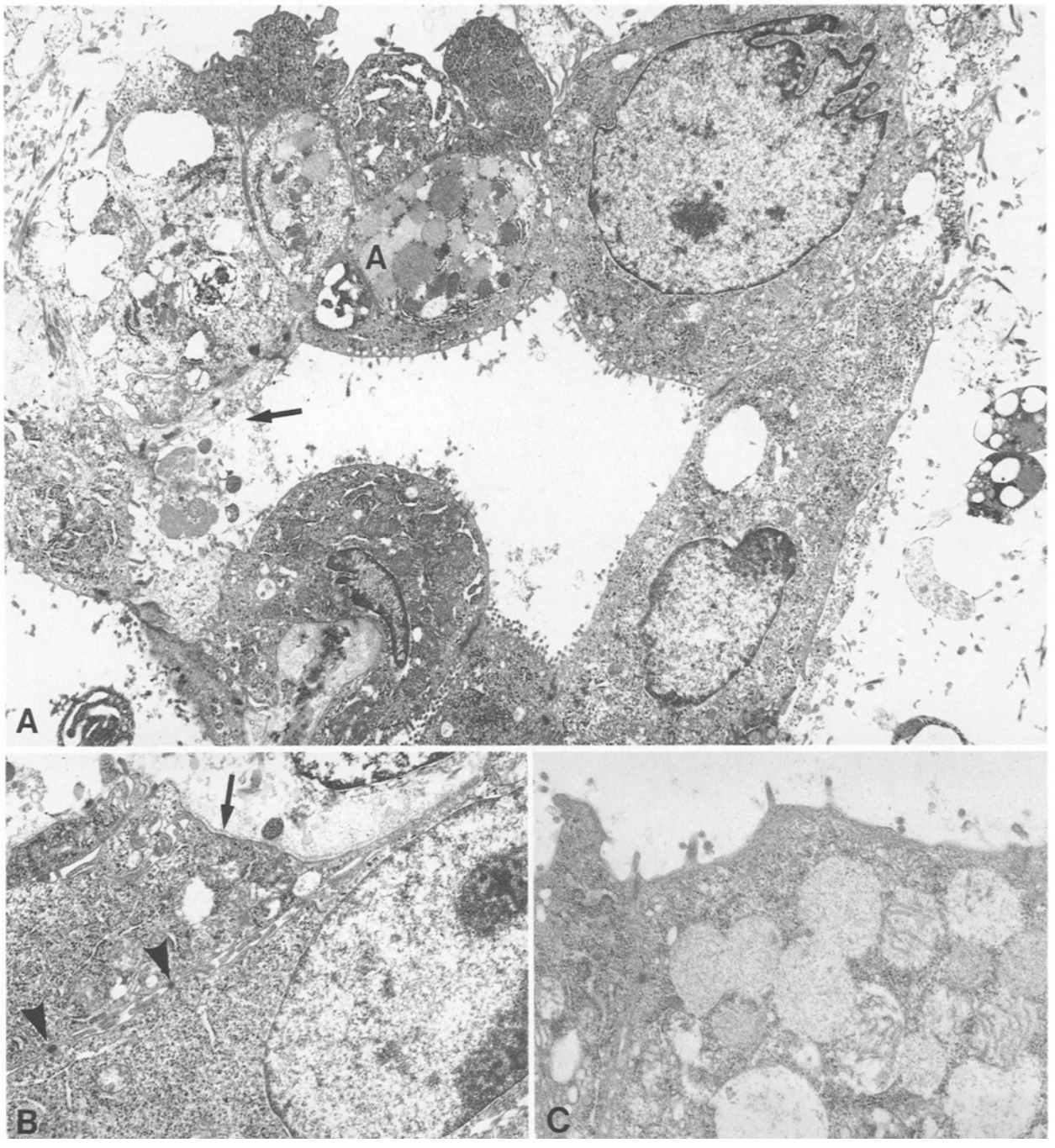

Fig. 6A–C. Organoids at 48 h of culture (no drug). **A** Persisting acinar cells (*A*) with zymogen granules reveal the terminal tubule origin of this organoid. Other cells are virtually devoid of granules. Much of the rough endoplasmic reticulum in all cells has disappeared. Some cells are autolysing (*arrows*). **B** Organoid cells maintain intercellular desmosomes (*arrowheads*) and focal basal lamina (*arrow*). **C** Higher magnification of zymogen-type granules in acinar cells in another organoid. UA & LC. **A** ×7800; **B, C** ×12500

had developed. BrdU incorporation into organoids gradually reduced but continued to day 30.

Electron microscopy. Twenty-four hours after the collagen gel matrices were established, the organoids had not fully recovered morphologically (Fig. 6A). However, lumens, complete with microvilli and tight junctions, and desmosomes were maintained (Fig. 6A). Compared to the in situ submandibular gland (Fig. 1) and the initially isolated organoids (Fig. 3), the major alteration was the marked reduction in rough endoplasmic reticulum in terminal tubular and acinar cells and the almost complete absence of secretory granules in the former cell type (Fig. 6A). Occasional cells contained secretory granules of acinar cell type (Fig. 6A, B), but by 48 h of culture even these were absent. Some organoids expressed basal lamina focally on cell surfaces adjacent to the collagen matrix (Fig. 6C).

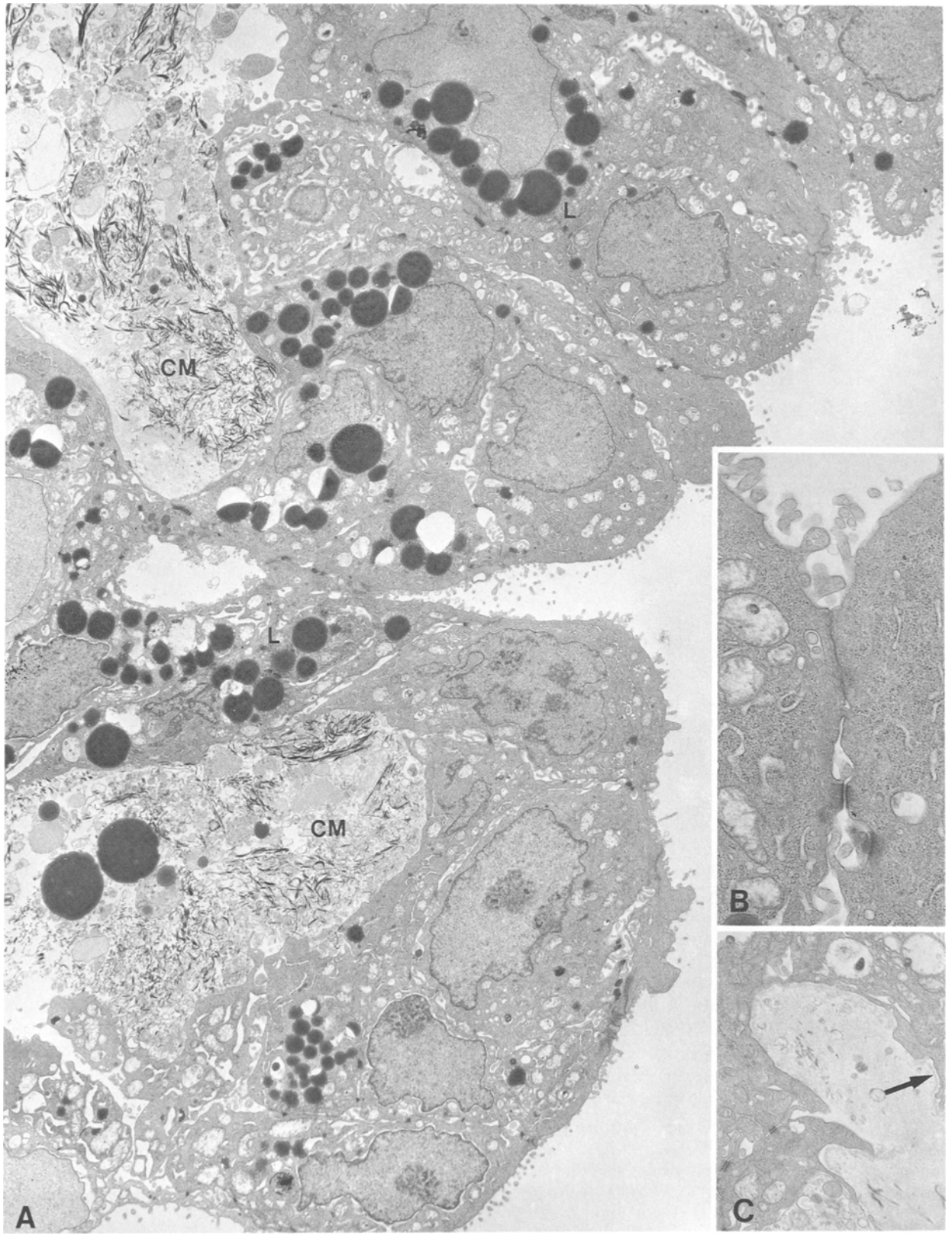

Fig. 7A–C. Organoid at day 5 of culture (no drug). **A** Cuboidal to low columnar cells, forming a glandular structure, lie within the collagen matrix (*CM*). Compared to the in situ cells (Fig. 1), rough endoplasmic reticulum continues to be reduced, secretory granules are absent and lipid droplets (*L*) are increased in number. **B** Glandular cells have well-formed apical junctional complexes. **C** Focal basal lamina (*arrow*) continues to develop at the collagen gel interface. UA & LC; **A** ×4000, **B** ×16500, **C** ×9800

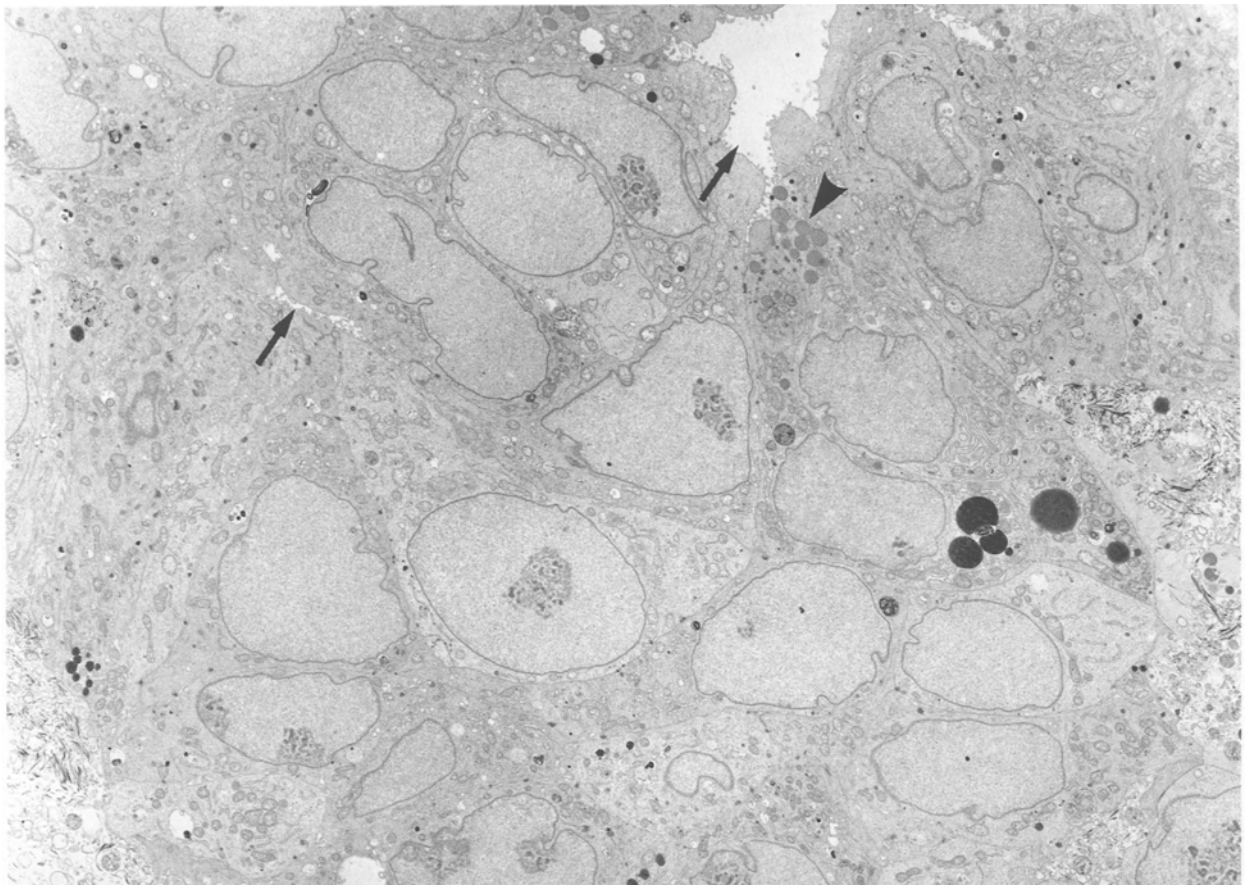

Fig. 8. Organoids at day 5 of culture (no drug). In some organoids, the cells are multilayered and lumens are small and often inconspicuous (*arrows*). Compared to Fig. 8, general cellular features are similar but lipid droplets are less frequent. An infrequent cell contains possible secretory granules (*arrowhead*). UA & LC, × 3300

After culture for 5 days, two types of growth pattern were evident (Figs. 7, 8). In one type (Fig. 7A), gland or duct-like structures occurred with one or two layers of cuboidal or polygonal-shaped cells joined by tight junctions and well-formed desmosomes (Fig. 7B). No apical secretory granules were present and rough endoplasmic reticulum was considerably reduced compared to the in situ gland (Fig. 1) or the initially isolated organoids (Fig. 3). The cells contained many lipid droplets and, very occasionally, basal lamina was developed (Fig. 7C). In other cases, the organoids were more solid in appearance with multiple small lumens (Fig. 8). The cells had similar nuclear and cytoplasmic features to those forming the glandular structures (Fig. 7), but there was less lipid and endoplasmic reticulum.

Organoids which had earlier been treated with isoproterenol tended to have only 1–2 layers of cells after 5 days in culture (Fig. 9A). General features (Fig. 9A), including microvilli and intercellular junctions (Fig. 9B, C), were similar to those in the organoids grown without the addition of isoproterenol (Fig. 7). In some organoids, salivary gland cells grew partially or completely across the central lumen (Fig. 9A). Occasionally, basally situated cells contained bundles of cytoplasmic filaments oriented in the long axis of the cell (Fig. 9A, B). No secretory products were evident. A few isoproterenol-treated organoids were more cellular, but still maintained multiple small lumens (Fig. 10). In these organoids, desmosomes, with comma-like tonofilaments, were more frequent and some cells, particularly at the periphery of the organoid, contained prominent accumulations of tonofilaments. Basal lamina partially encircled this type of organoid (Fig. 10).

After culturing the organoids in collagen gel matrix to day 10, there were few if any cytological changes, and secretory granules or other markers of differentiation were not developed. Ultrastructurally, these maintained evidence of glandular differentiation, but where the cells became increasingly layered, some features of squamous differentiation (such as in Fig. 10) were evident.

Discussion

The biological pathways that result in the development of salivary gland neoplasms are not known. Despite this, a number of hypotheses as to the cell types involved in the induction of these neoplasms, the differentiation pathways for evolving neoplastic population(s), and the

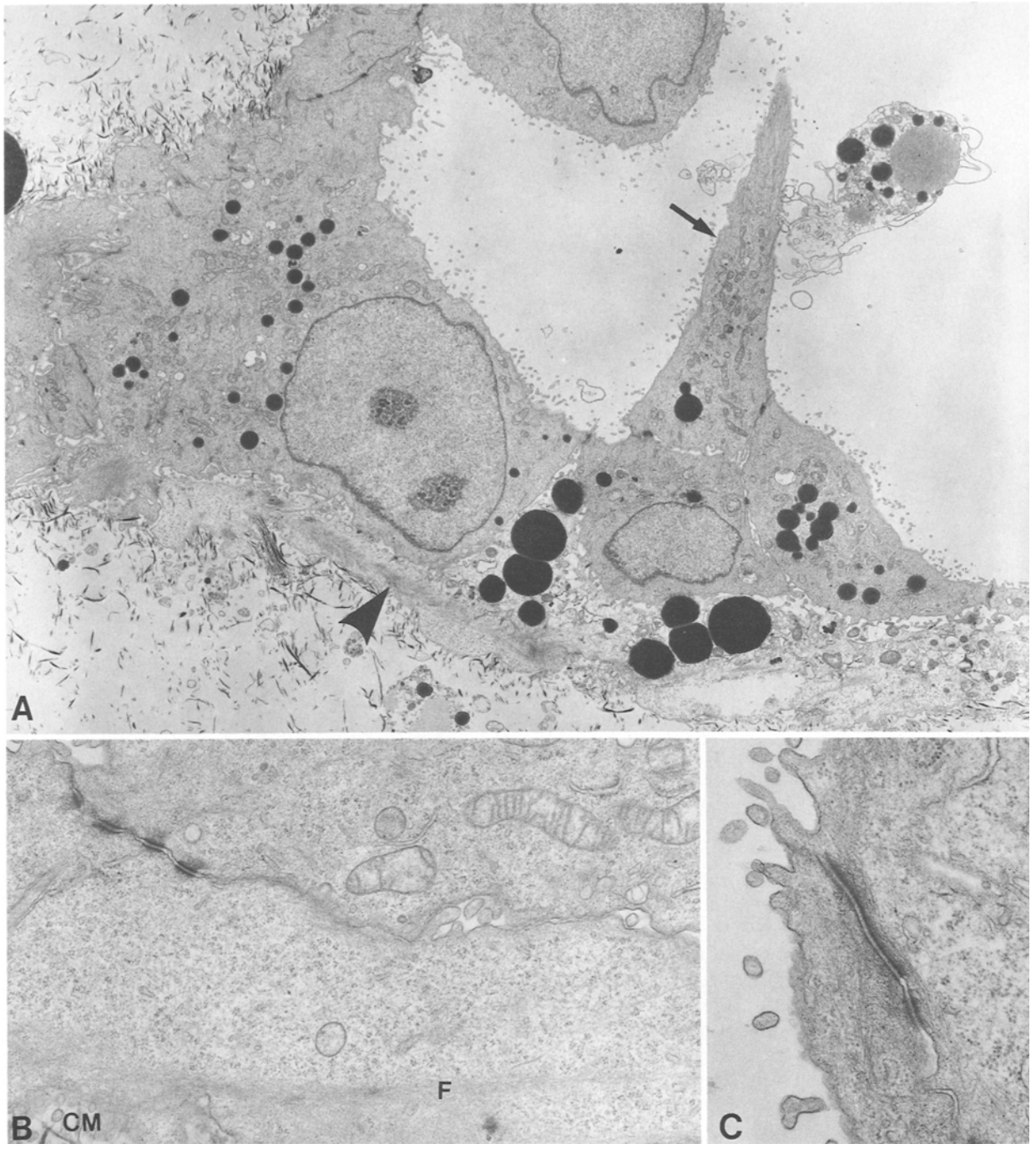

Fig. 9A–C. Organoid culture at day 5 (isoproterenol-treated). **A** Glandular cells are structurally similar to those in Figs. 7 and 8, but some cells (*arrow*) are beginning to grow across the lumen. Some basally situated cells contain accumulations of filaments (*arrowhead*). **B** Adjacent to the collagen matrix (*CM*), there are aggregates of well-aligned filaments (*F*). Cells are joined by typical desmosomes with complements of intermediate filaments (*arrows*). **C** Specialized apical junctions continue to be maintained in these organoids. UA & LC, **A** ×3300, **B** ×15400, **C** ×17500

biological processes resulting in the characteristic histology of salivary gland tumours have been advanced (Regezi and Batsakis 1977; Batsakis 1980; Dardick and van Nostrand 1987; Dardick et al. 1990). Several of the concepts underlying these hypotheses are testable, but require the development and standardization of an animal model for their experimental evaluation. Human salivary gland tumours display a complex interplay of luminal and myoepithelial or myoepithelial-like cells and a wide spectrum of differentiation of these cells resulting in an impressive range of morphology. As an in vitro experimental model, a culture system that uses all cell types and maintains the three-dimensional structure of the in vivo gland would therefore seem most appropriate.

In this study organoids prepared from the submandibular glands of 8-day-old rats were representative of

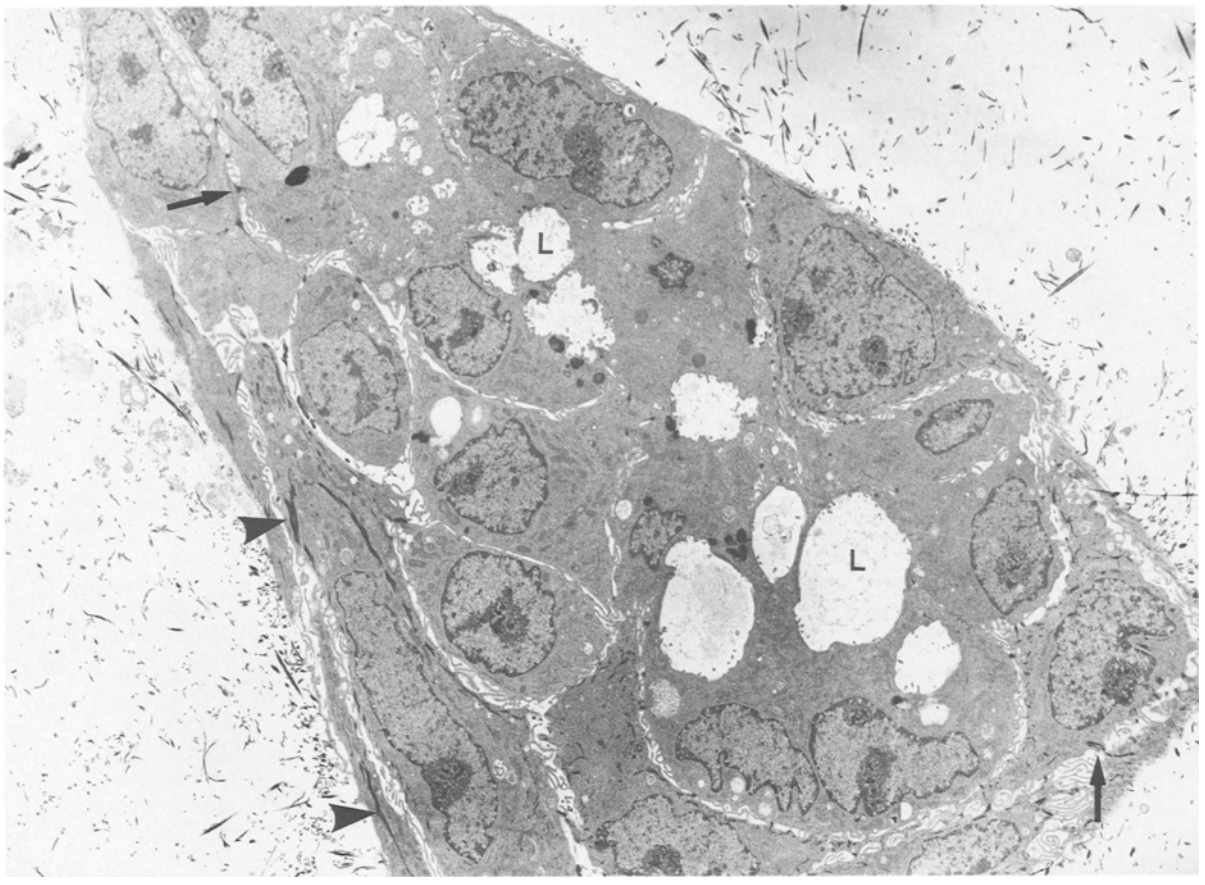

Fig. 10. Organoid culture at day 5 (isoproterenol-treated). Certain cellular organoids maintain glandular lumens (*L*), but some peripheral cells have developed intercellular bridges complete with tonofilament-associated desmosomes (*arrows*) and also contain extensive accumulations of tonofilaments (*arrowheads*). UA & LC, ×3300

the parent tissue and grew satisfactorily in the collagen gel for up to 30 days. Growth appeared to peak between 5 and 10 days and thereafter, cell death exceeded cellular proliferation and the number of viable colonies present diminished. However, BrdU labelling of cultures indicated that cycling cells were still present after 30 days of culture, particularly among the squamous-like cells (Fig. 10) that grew out over the surface of the gel. Markers for acinar cells such as specific secretory granules and extensive endoplasmic reticulum, which had persisted after isolation, were infrequently observed by 24 h of culture and, subsequently, acinus cell-type granules completely disappeared. This process was paralleled by similar alterations in terminal tubule cells, so that the organoids eventually became composed entirely of a uniform population of rather primitive-looking cells which were still capable of dividing.

The addition of isoproterenol to the cultures resulted in an alteration in growth pattern, with earlier and more abundant peripheral outgrowth from treated organoids compared to untreated controls, and a marked tendency for the proliferating cells to grow out over the surface of the gel with squamous differentiation. However, examination of cultures grown for 48 h, 5 and 10 days and post-fixed in osmium so that the colonies of cells were clearly visible as black areas within the gels suggested that isoproterenol had not stimulated additional growth but had merely shifted the peak of proliferation so that rapid growth was induced earlier in treated compared with untreated cultures. It has previously been shown that a single intraperitoneal injection of isoproterenol will cause a synchronized burst of one round of mitosis in the salivary glands of adult rats, and then the gland parenchyma becomes quiescent again (Baserga 1970). Such a mechanism may well be responsible for the isoproterenol-induced early burst of growth in this study, as well as the morphological differences in colony organization in treated cultures compared to controls. In turn, the drug treatment may have been responsible for the resulting reduced capacity for further proliferation.

The present results, which are preliminary in nature, suggest that primary cultures of salivary gland organoids in a three-dimensional matrix may prove useful as a model for investigating the pathobiology of the salivary gland. Chronic sialadenitis in human submandibular gland (Matthews and Dardick 1988) causes acinar cell changes similar to those described above for the rat salivary gland organoids, while marked cytological alterations also occur in the epimyoepithelial islands of benign lymphoepithelial lesions (Caselitz et al. 1986; Dardick et al. 1988). In addition, anoxia of the rat subman-

dibular gland rapidly leads to squamous metaplasia of both acinar and myoepithelial cells (Dardick et al. 1985), a cellular modification readily reproducible in this system during the later stages of culture.

Further studies will include both phenotypic characterization of the cultured cells and manipulation of the culture conditions, since conditions optimal for proliferation of epithelial cells differ from those which promote differentiation (Freshney 1987). Altering the formulation of the growth medium used at different stages of culture, and the addition of chemicals such as sodium butyrate, dibutyryl cyclic adenosine 3′,5′-monophosphate or 12-*O*-tetradecanoyl-phorbol-13-acetate may allow terminally differentiated cells to predominate in the cultures with time. This has been achieved with cultured monolayers of epithelial cells derived from a human salivary gland adenocarcinomas or similar tumour cells grown in nude mice (Hayashi et al. 1985, 1987; Azuma et al. 1986; Yoshida et al. 1986). Various hormones have also been used to alter patterns of differentiation in salivary gland tissue in vitro (Maruyama et al. 1982).

An unresolved issue in salivary gland neoplasms on which the results of this study may have some bearing is whether or not oncogenesis is solely dependent on stem ("reserve") cells or whether this process can be initiated in fully differentiated cells (Batsakis 1980). A function of terminal tubules is differentiation into acinar cells (Chang 1974), a process clearly underway in the 8-day-old rat submandibular gland (Fig. 1). It has been suggested that acinar cells, being terminally differentiated, cannot be considered potentially oncogenic cells unless they have previously undergone dedifferentiation – a cellular event considered unlikely (Batsakis 1980; Batsakis et al. 1989). The results of this in vitro study indicate that cellular events analagous to dedifferentiation can occur since organoids that initially contained all the cell types present in the mature gland produced large clusters of cells with no specific features within 24–48 h.

Regenerative processes in the pancreas have been suggested as a model for stem cell function in the salivary gland and a parallel system for the process of tumour induction (Batsakis et al. 1989). However, recent evidence from experimental studies of proliferative capacity during regeneration of the adult rat pancreas assigns the major role of precursor cell to the acinar cell, illustrates that dedifferentiation can occur in these cells and, significantly, that the acinar cell is the most at risk when exposed to carcinogens (Rao et al. 1990). Although it is possible that in the present study the death of differentiated cells and/or selective overgrowth of undifferentiated cells during culture influenced the final population, both the rapidity with which the terminal tubule and acinar cells altered, as well as the uniform nature of the outgrowth from them, suggests that all cell types present in the isolated organoids were acting as stem cells. Whether any or all of such cells can be induced to redifferentiate requires further study.

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