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Cell proliferation is not required for the initiation of early cleft formation in mouse embryonic submandibular epithelium *in vitro*

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Summary

An X-ray irradiation method was employed to analyse the role of cell proliferation *in vitro* in the cleft formation of mouse embryonic submandibular epithelium at early stages. When the mid 12-day gland was exposed to 200 rad of X-rays, the growth was severely retarded. In contrast, late 12-day and early 13-day glands grew apparently in a normal fashion, as did the control gland, for up to 40 h. In either case, they formed shallow clefts within 10 h of culture. With 1000 rad irradiation, the mid 12-day gland did not grow at all, but formed clefts within 20 h of culture followed by a rapid degeneration. Under the same conditions, the growth of the late 12-day gland, which was at the stage just before branching, was retarded until 10 h of culture, followed by a slight increase in epithelial size, but cleft formation was also observed within 6–10 h, as in the control gland. When exposed to a dose of 1000 rad of X-rays, the early 13-day and the late 12-day glands exhibited similar radiosensitivity; the initial narrow clefts in the epithelium deepened and new clefts began to form within 6–10 h of culture. [^{3}H]thymidine incorporation studies revealed that a dose of 1000 rad reduced DNA synthesis of mid and late 12-day glands by 72 and 65 %, respectively. Histological examination of X-irradiated late 12-day gland showed that mitotic figures were rarely seen in the epithelium at 6 h of culture. Aphidicolin, a specific inhibitor of DNA synthesis, could not halt the cleft formation of the late 12-day gland. In this experiment 89 % of DNA synthesis was inhibited. Treatment of an X-ray irradiated late 12-day gland with aphidicolin blocked 92 % of the DNA synthesis, but did not prevent cleft formation taking place. These results indicate that neither cell division nor DNA synthesis, is required for the initiation process of the cleft formation of the mouse embryonic submandibular epithelium at early morphogenetic stages *in vitro*.

Key words: mouse, submandibular gland, cleft formation, branching, aphidicolin, X-ray irradiation, cell proliferation, mitosis.

Introduction

The branching morphogenesis of the mouse embryonic submandibular gland has long been analysed by focusing on how the epithelium interacts with the mesenchyme and how the epithelium changes its shape to achieve characteristic branching. These studies, including the pioneering works by Borghese (1950) and Grobstein (1954), have indicated that the proper morphogenesis is the consequence of complex events: cell proliferation (Bernfield, Banerjee & Cohn, 1972; Bernfield & Banerjee, 1982; Lawson, 1974), epitheliomesenchymal interactions through extracellular matrix components (Grobstein & Cohen, 1965; Kallman & Grobstein, 1965; Bernfield, 1970; Bernfield & Wessells, 1970; Banerjee, Cohn & Bernfield, 1977; Cohn, Banerjee & Bernfield, 1977; Bernfield & Banerjee, 1982; Spooner & Faubion, 1980; Spooner, Bassett & Stokes, 1985) and cell shape change of the epithelium mediated by intracellular

microfilaments (Spooner & Wessells, 1972; Ash, Spooner & Wessells, 1973). Recently, these studies have been evaluated by Goldin (1980) and Ettensohn (1985).

In order to understand the mechanism of the epithelial branching induced through such complex events, it is necessary to ask the role of each individual event separately. By using a collagenase inhibitor from bovine dental pulp (Kishi & Hayakawa, 1984) and *Clostridial* collagenase freed from proteinase and glycosaminoglycan-degrading activities, we have already concluded that collagen plays a crucial role in the initiation process of cleft formation (Nakanishi, Sugiura, Kishi & Hayakawa, 1985, 1986*a*,*b*). Scanning electron microscopic studies have also indicated that collagen-like fibres are preferentially localized at the sites where branching takes place (Nakanishi, Sugiura, Kishi & Hayakawa, 1986*c*). Furthermore, it is of importance to note that the mesenchyme can determine the epithelial curvature irrespective of the stage of the epithelium used (Nogawa, 1983). By summarizing these observations, a mesenchymal traction hypothesis has been proposed as one of the possible models for the initial cleft formation, in which the mesenchyme, containing collagen fibres, deforms the epithelial surface (Nakanishi *et al.* 1986*c*). If this is the case, it is reasonable to assume that cell proliferation in the epithelium is not required for cleft initiation.

It has also been shown in histological sections that mitotic cells are often seen in the epithelium at the base of clefts of early and mid 13-day glands (Nakanishi & Nogawa, unpublished observation). The mitotic behaviour of the epithelium cells seems likely to affect the morphogenesis of the distal epithelial bulb.

It is now well established that X-ray irradiation suppresses cell proliferation through inhibition of DNA synthesis and mitosis (Elkind & Whitmore, 1967; Altman, Gerber & Okada, 1970). We therefore employed an X-ray irradiation technique instead of colchicine, an inhibitor of cell division, because the drug was severely toxic in the culture conditions we used for early salivary glands; the lobules of 12-day and 13-day glands became rounded within 6 h of their exposure to $0{\cdot}1$–$1{\cdot}0\,\mu g\,ml^{-1}$ of colchicine, concentrations previously used for the examination of cleft formation in mid 13-day glands (Spooner & Wessells, 1972). Moreover, narrow, sharp clefts of late 13-day gland epithelium were lost under the same conditions. It was also essential for us to avoid the difficulty in interpretating experimental results which would be raised by the fact that microtubule-disrupting reagents are known to enhance the rounding up of cells and promote their detachment from substrata containing collagens (Bell, Ivarsson & Merrill, 1979).

The object of the present study was to assess the role of epithelial cell proliferation during the initial phase of branching morphogenesis. Synthesis of DNA, in addition to cell division, was also evaluated by using aphidicolin, which had been successfully utilized for the morphogenetic study on supernumerary bud induction in mouse embryonic lung (Goldin, Hindman & Wessells, 1984). Since it had already been shown that cell proliferation in the submandibular epithelium is much more rapid than that in the mesenchyme (Bernfield *et al.* 1972; Bernfield & Banerjee, 1982), it was reasonable to assume that treatment with X-ray and aphidicolin would have most effect on the activity of epithelial cells.

Materials and methods

Materials

Aphidicolin, a specific inhibitor of DNA polymerase α of eukaryotic cells (Ikegami, Taguchi, Ohashi, Nagano & Mano, 1978; Longiaru, Ikeda, Jarkovsky, Horwitz & Horwitz, 1979; Huberman, 1981), was kindly donated by Dr T. Okazaki, Institute of Molecular Biology, Nagoya University. [^{3}H]thymidine ($23{\cdot}8\,Ci\,mmol^{-1}$) was obtained from New England Nuclear Corporation, Boston, Massachusetts.

Embryos

12-day and 13-day embryos were obtained from DDY strain mice. The day of discovery of the vaginal plug was designated as day 0.

Organ culture and labelling

Embryonic salivary glands were dissected out from 12-day and 13-day embryos in Tyrode's solution. Glands at three different stages were used: mid 12-day glands having distal epithelial bulb without any indication of clefts, late 12-day glands with first signs of indentations and early 13-day glands with definite clefts. Organ culture was routinely performed on ultrathin Millipore filter (THWP, $0{\cdot}45\,\mu m$ pore size) assemblies (Banerjee *et al.* 1977) in BME medium supplemented with ascorbic acid ($50\,\mu g\,ml^{-1}$) and 10 % fetal calf serum in a 37 °C incubator with a humidified atmosphere of 95 % air/5 % CO_2. When aphidicolin was used, varying amounts of the drug were included in the medium at the onset of culture. Labelling medium contained $1{\cdot}0\,\mu Ci\,ml^{-1}$ [^{3}H]thymidine for experiment 1 and $0{\cdot}95\,\mu Ci\,ml^{-1}$ for experiment 2 (see Table 1) and glands were cultured for 6 h. After incubation, tissues were washed four times with 10 % trichloroacetic acid and the precipitates were dissolved in $0{\cdot}2$ M-NaOH. DNA synthesis was assessed by counting the radioactivity of acid-insoluble materials.

Living cultures of salivary glands were monitored by taking photographs at appropriate intervals on an Olympus light microscope BH-2 equipped with an automatic exposure unit. For the semiquantitative assessment of epithelial growth, the contour of lobules of photographs was

traced in pencil on a transparent paper, which was copied to a heavier paper. The areas covered with lobules were determined by cutting out and weighing the pieces of paper and were plotted as in Fig. 4. To normalize the differences in initial epithelial sizes, a value, the 'relative growth rate' (area of lobules at the indicated time divided by the area of lobule of 1 h culture), was introduced. The area of stalk region was not considered because the increment in cell populations of the stalk region after cultivation seemed to be due to the migration of oral epithelial cells attached to the bottom end of stalk, as well as due to cell proliferation (Nakanishi & Nogawa, unpublished observation), as suggested for mouse mammary gland (Hogg, Harrison & Tickle, 1983).

Irradiation of salivary glands with X-rays

Embryonic rudiments were irradiated with X-rays (Hitachi MBR 1520R) in a plastic tube containing BME medium without fetal calf serum at a dose rate of 250 rad min^{-1}, 150 kVp and 20 mA, with 0·1 mm Cu and 0·5 mm Al filtration (HVL 0·9 mm Al) at 24°C. The X-ray dose was measured by a Victoreen R meter and the absorbed dose was estimated from the exposure using the factor 0·94/R (ICRU report 10b 1962, 1964). The cultivation of glands began at 1 h after the irradiation.

Histology

Specimens were treated in Bouin's fixative,dehydrated in ethanol, embedded in paraffin and sectioned into 4 μm thickness. The sections were stained with haematoxylin.

Results

Abilities of glands irradiated with X-rays to form clefts and grow

Mid 12-day gland

Fig. 1 clearly shows that X-irradiation had severe effects on the growth of the mid 12-day gland even with a dose of 200 rad (Fig. 1, middle row), as compared with that of the control gland (Fig. 1, top row). The X-irradiated lobule, however, branched normally. With a higher dose of 1000 rad, the growth of the treated gland halted totally, as is evident in

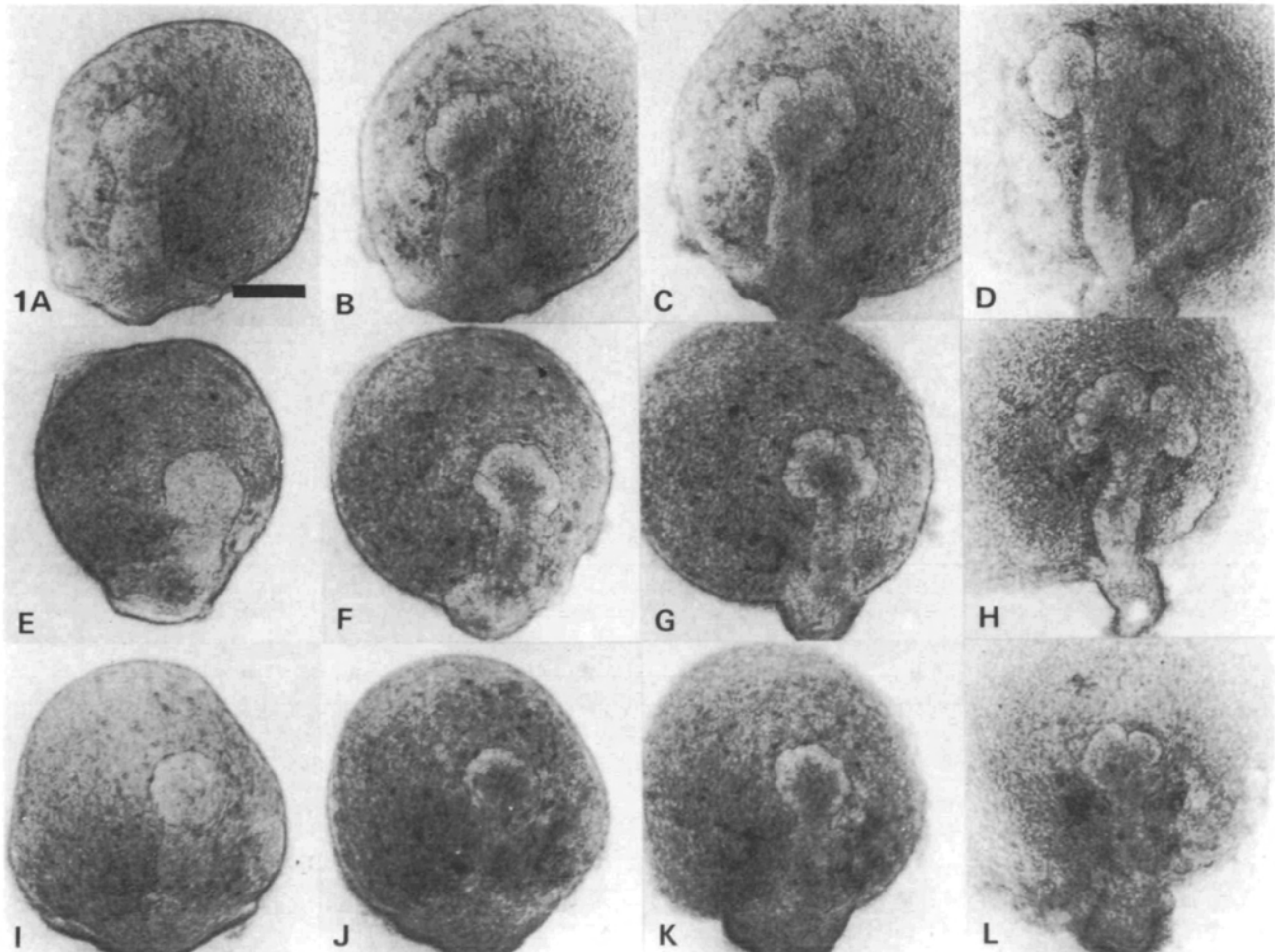

Fig. 1. Effects of X-ray irradiation on submandibular gland morphogenesis. A control mid 12-day gland is shown successively at (A) 1 h, (B) 6 h, (C) 10 h and (D) 27 h of culture. Glands irradiated with a dose of 200 and 1000 rad are shown successively at (E, I) 1 h, (F, J) 6 h, (G, K) 10 h and (H, L) 27 h, respectively. Bar, 0·2 mm.

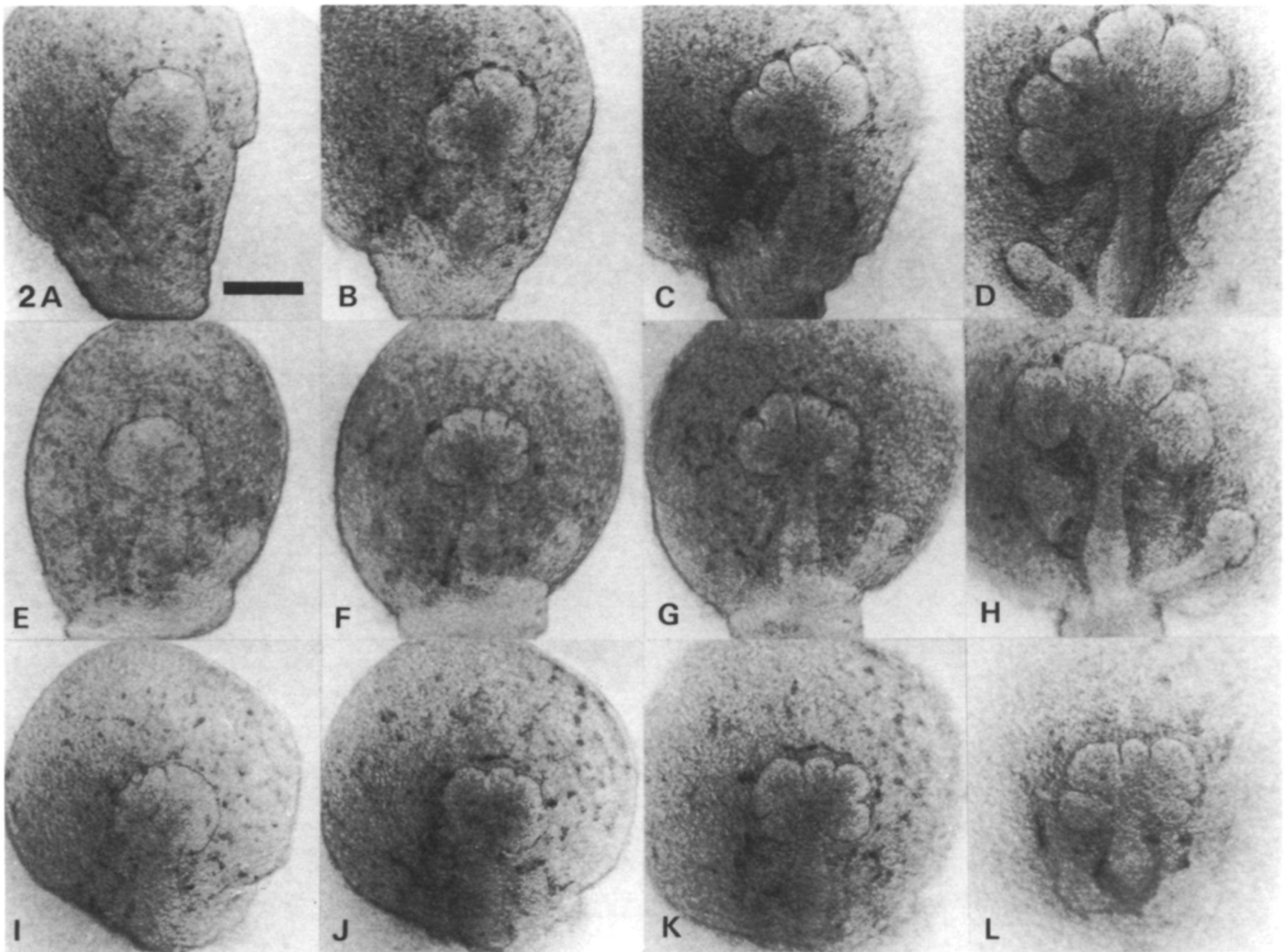

Fig. 2. Effects of X-ray irradiation on submandibular gland morphogenesis. A control late 12-day gland is shown successively at (A) 1 h, (B) 6 h, (C) 10 h and (D) 27 h of culture. Glands irradiated with a dose of 200 and 1000 rad are shown successively at (E, I) 1 h, (F, J) 6 h, (G, K) 10 h and (H, L) 27 h, respectively. Bar, 0·2 mm.

Fig. 1 (bottom row) and then degenerated. [^{3}H]thymidine incorporation into acid-insoluble materials during the initial 6 h of culture indicated that 72 % of DNA synthesis was inhibited (Table 1), consistent with earlier reports that cells irradiated with 1–3 krad of X-rays generally have only one third of the DNA-synthesizing activities (Tolmach & Jones, 1977; Painter, 1986). Even in this case, cleft formation, though delayed, was observed (Fig. 1, bottom row).

Late 12-day and early 13-day glands

In contrast to the mid 12-day gland, late 12-day and early 13-day glands were more resistant to X-ray irradiation. When a dose of 200 rad was given, their growth was almost the same as the control and the formation of clefts was unimpaired (Figs 2, 3, top and middle rows). However, when these glands were exposed to 1000 rad of X-rays, a severe growth retardation was observed (Figs 2, 3, bottom row) and [^{3}H]thymidine incorporation was 35 % of controls for the initial 6 h of culture (Table 1).

In order to estimate more quantitatively the growth of the epithelial lobules cultured on Millipore filter assemblies, the enlargement of late 12-day gland epithelium, as a typical example, was expressed as areas covered with lobules. As Fig. 4 indicates, the epithelium of control glands enlarges slowly for the initial 10 h of culture, then exponentially for up to 40 h. Since histological studies showed that sizes of cultured lobular epithelial cells were not obviously different from those of intact late 12-day glands, it was reasonable to believe that such enlargement in area reflected epithelial cell proliferation. No initial enlargement of late 12-day and early 13-day epithelia irradiated with 1000 rad of X-rays was observed (Figs 2, 3, bottom row). Rather, there was a slight reduction in area at 6 h of culture (Fig. 4). This was in contrast to the results obtained using aphidicolin, in which a small, though significant increase in area was observed when DNA synthesis was reduced by 89 % by 2·0 μg ml^{-1} of the drug (Table 1 and see also Figs 4, 5E–H). This could be attributed to an increase

in cell number due to the subsequent division of cells at the G_2 phase when they were exposed to aphidicolin. A later enlargement of epithelial areas with 1000 rad of X-rays (Fig. 4) was probably due to a cell division after the residual synthesis of DNA (Table 1). Judging from the degrees of inhibition of DNA synthesis and lobular enlargement, late 12-day and early 13-day glands were less sensitive than the mid 12-day glands towards irradiation by X-rays.

However, under these growth conditions, the epithelial branching of late 12-day gland went on within 6 h of culture, just as in the lobule of the control gland (Fig. 2, top and middle rows). In addition, as clearly shown in Fig. 3, the initial clefts found in the gland at the onset of culture deepened, in the same way as in the control during 6 h of culture after X-irradiation with 1000 rad. These results, taken together with those from the mid 12-day gland, demonstrate that cell division, irrespective of epithelial or mesenchymal, as well as lobular enlargement, is not directly related to the initiation and further deepening of clefts in the epithelium at stages later than mid 12-day.

Abilities of glands treated with aphidicolin and X-rays to grow and form clefts

As described above, cleft formation of the lobular epithelium of glands at mid and late 12-day and early 13-day was not inhibited by X-irradiation with a dose of 1000 rad, which clearly prevented most cell division (see below). In these cases, however, approximately 30 % of DNA-synthetic activities was still maintained. Further experiments were therefore necessary to remove the possibility that DNA synthesis was essential for cleft initiation and its deepening.

Fig. 4 shows that as the concentrations of aphidicolin increase, the enlargement of the lobular epithelium is reduced and there is the strongest depression in growth at $2{\cdot}0\,\mu g\,ml^{-1}$. Since no enlargement was seen after 24 h of culture at that concentration, concentrations higher than $2{\cdot}0\,\mu g\,ml^{-1}$ were not examined. [^{3}H]thymidine incorporation studies

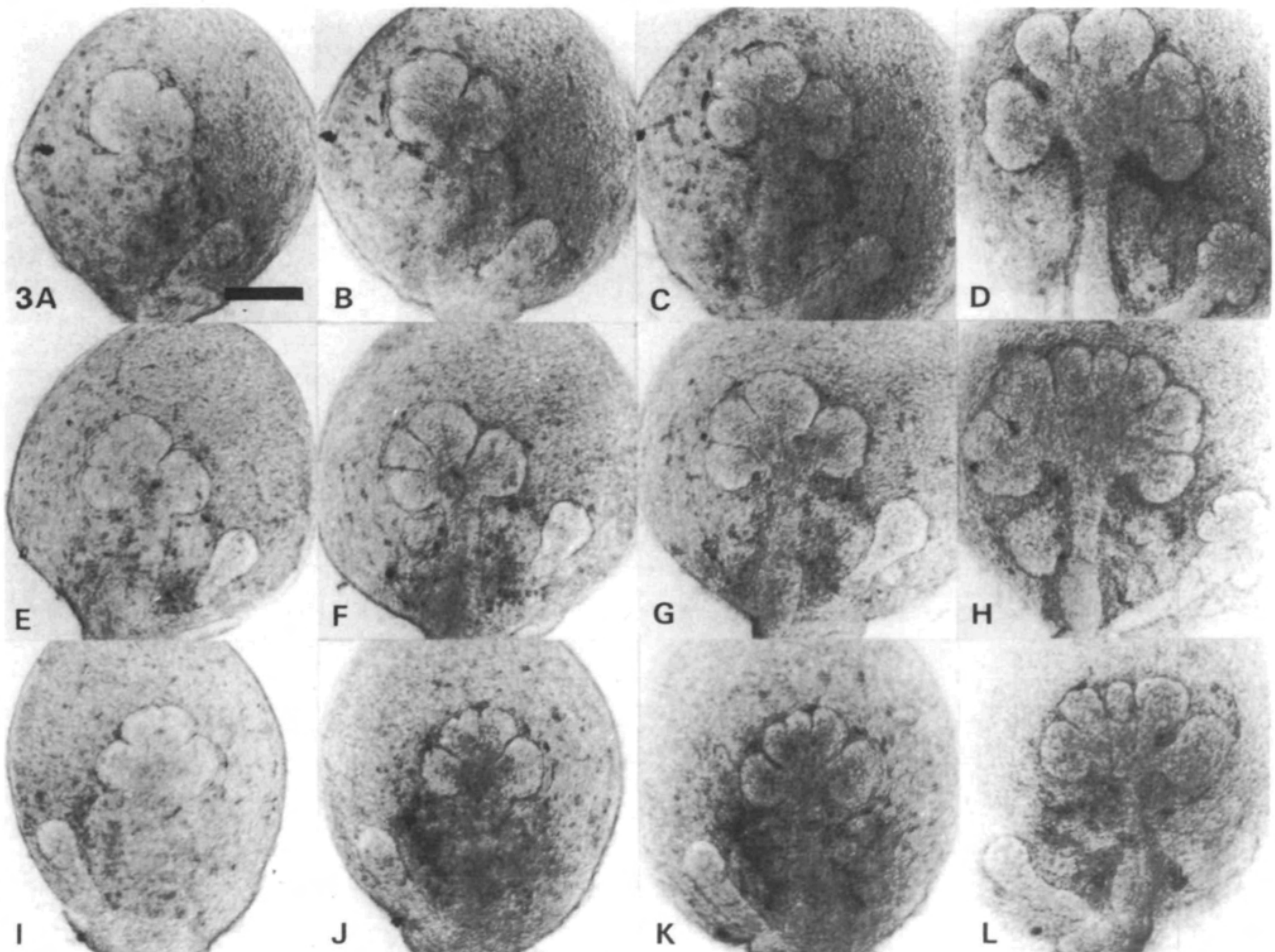

Fig. 3. Effects of X-ray irradiation on submandibular gland morphogenesis. A control early 13-day gland is shown successively at (A) 1 h, (B) 6 h, (C) 10 h and (D) 27 h of culture. Glands irradiated with doses of 200 and 1000 rad are shown successively at (E, I) 1 h, (F, J) 6 h, (G, K) 10 h and (H, L) 27 h, respectively. Bar, 0·2 mm.

Table 1. *Effects of aphidicolin (2·0 μg ml^{-1}) and X-ray irradiation (1000 rad) on [^{3}H]thymidine incorporation into acid-insoluble materials of 12-day submandibular glands*

Treatment	Number of glands used	Radioactivity (dissints min^{-1}/gland)	Relative activity (%)
Experiment 1			
middle 12-day:			
control	19	1115	100
+X-ray	24	315	28·3
late 12-day:			
control	20	1430	100
+X-ray	20	498	34·8
Experiment 2			
late 12-day:			
control	21	1093	100
+aphidicolin	21	121	11·1
+aphidicolin +X-ray	22	90	8·2

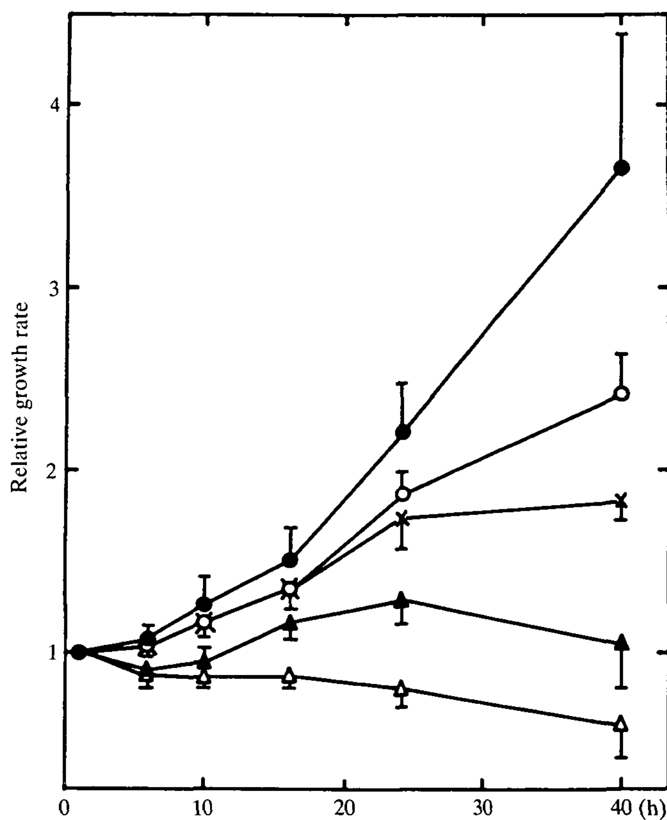

Fig. 4. Effects of aphidicolin and X-ray irradiation on the epithelial growth of late 12-day glands. ●, control glands; ○, glands treated with aphidicolin (0·2 μg ml^{-1}); ×, glands treated with aphidicolin (2·0 μg ml^{-1}); ▲, glands irradiated with X-rays at a dose of 1000 rad; △, glands treated with aphidicolin (2·0 μg ml^{-1}) after X-ray irradiation of 1000 rad. *Relative growth rate* was calculated by dividing the area of lobule at indicated times by that of lobule of 1 h culture as described in Materials and Methods. Each point represents a mean ±S.D. of more than five glands.

indicated that aphidicolin, at 2·0 μg ml^{-1} in the culture medium, inhibited 89 % of DNA-synthesizing activity of late 12-day gland for the initial 6 h of culture (Table 1), but cleft formation was not inhibited at all (Fig. 5, middle row).

Finally, X-irradiated late 12-day glands (1000 rad dose) were exposed to 2·0 μg ml^{-1} of aphidicolin and examined for their abilities to form clefts and to synthesize DNA. Incorporation of [^{3}H]thymidine into the gland during the initial 6 h of culture was only 8 % of controls, indicating that the remaining DNA-synthesizing activity after X-irradiation was further inhibited by the drug. Nevertheless, clefts were formed as after irradiation alone: compare the bottom row of Fig. 5 with those of Figs 1–3. These results, taken together, indicate that cleft formation is not dependent on cell division or on DNA synthesis.

Histological examination of glands irradiated with X-rays

Late 12-day glands that were exposed to 1000 rad of X-rays and cultured for 6 h, were compared with normally cultured glands. Mitotic figures (in metaphase and anaphase) were rarely found in 4 μm sections of irradiated glands compared with those of controls. Thus, 3·0 ± 0·9 mitotic figures per 100 epithelial cells on average were detected in the control epithelium of 6 h of culture and 0·4 ± 0·4 in the X-irradiated epithelium. Additionally, considerable numbers of cells in the outer layer of the mesenchyme and of epithelial cells inside the lobule and stalk became necrotic. Only a few mitotic cells were observed in the mesenchyme of the control gland, consistent with the earlier finding that most of ^{3}H-grains found after incorporation of [^{3}H]thymidine into 13-day glands are localized in the lobular epithelium (Bernfield *et al.* 1972; Bernfield & Banerjee, 1982). These results indicate that, although X-

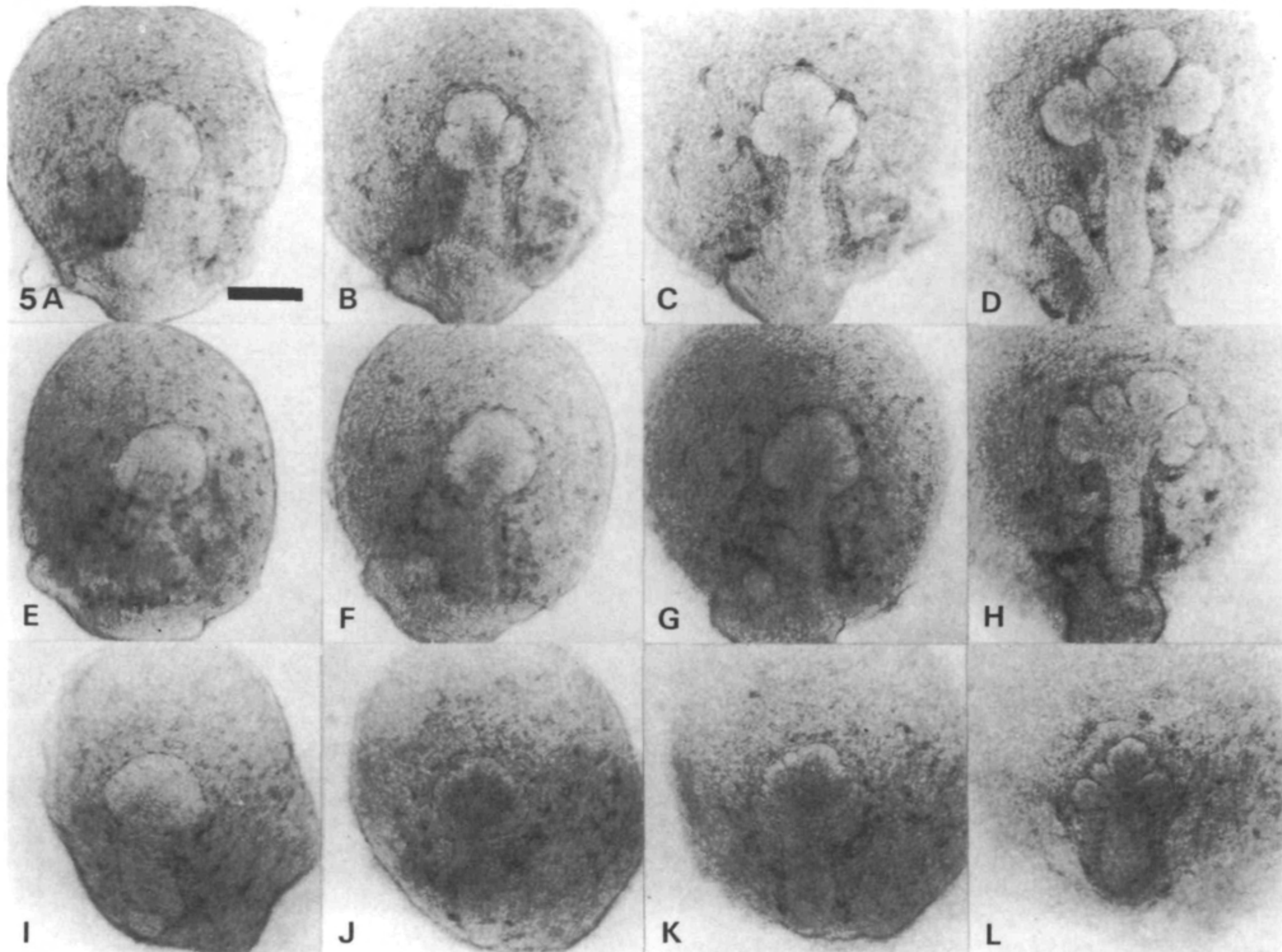

Fig. 5. Effects of aphidicolin and X-ray irradiation on submandibular gland morphogenesis. A control late 12-day gland is shown successively at (A) 1 h, (B) 6 h, (C) 10 h and (D) 24 h of culture. An aphidicolin-treated gland ($2{\cdot}0\,\mu g\,ml^{-1}$) is shown successively at (E) 1 h, (F) 6 h, (G) 10 h and (H) 24 h of culture. An aphidicolin-treated gland ($2{\cdot}0\,\mu g\,ml^{-1}$) after X-ray irradiation (1000 rad) is shown successively at (I) 1 h, (J) 6 h, (K) 10 h and (L) 24 h. Bar, 0·2 mm.

irradiation inhibited cell division, resulting in substantial cell death, nevertheless, normal branching patterns were observed in the glands exposed to such an extremely high dose of X-rays.

Discussion

X-ray irradiation techniques have been useful for the investigation of embryonic development (Russell & Russell, 1954) but have rarely been used for morphogenetic studies of embryonic organs. It is now well known that a dose of 100–200 rad of X-rays causes a delay in cell division of several hours and a dose of more than 1000 rad gives severe double strand breakage of DNA, leading not only to a delay in mitosis but also to a failure in further cell proliferation, depending on the types of cells examined (Elkind & Whitmore, 1967; Altman *et al.* 1970).

The results obtained in the present study were very simple, but important. A dose of 200 rad of X-rays appeared to bring about a delayed cell division and 1000 rad additionally impaired the machinery for further cell proliferation (Figs 1–3, middle and bottom rows). It was thus concluded that inhibition of cell division and of DNA synthesis could not halt the branching of mouse submandibular glands *in vitro* at the mid and late 12-day stages.

It is, therefore, reasonable to assume that the role of cell proliferation is, at least in part, to provide the epithelial lobule with a necessary volume for branching. In this respect, it is important to note that mesenchyme can make clefts only on a lobule that is larger than the threshold size (Nogawa, 1983). Thus, in recombination experiments *in vitro*, 14-day lobules grew, but did not form clefts, in the presence of 13-day mesenchyme, while 14-day mesenchyme was able to induce shallow clefts after a similar expansion of 14-day lobules (see fig. 5 in Nogawa, 1983), suggesting that the size of the lobule necessary for branching depends on the stages of the mesenchyme used. This

stage dependency of the mesenchyme for the ability to induce clefts might reflect a shaping (deforming) force of mesenchyme previously postulated by Nakanishi *et al.* (1986*c*). This hypothesis is principally based on the fact that several types of fibroblastic and mesenchymal cells contract collagen gels in culture (Bell *et al.* 1979; Steinberg, Smith, Colozzo & Pollack, 1980; Harris, Stopak & Wild, 1981; Stopak & Harris, 1982), together with our previous findings that collagen plays a crucial role in the initiation of cleft formation (Nakanishi *et al.* 1986*a*,*b*,*c*).

These considerations, together with the results presented in this paper, support a hypothesis that cleft formation is a consequence of a balance between the shaping force of the mesenchyme and the expansion of the epithelium encircled with basal lamina. For example, both a possible increase of the shaping force by the treatment of gland with a collagenase inhibitor (Nakanishi *et al.* 1985, 1986*a*,*b*) and a decrease in the lobular expansion by X-irradiation or aphidicolin treatment in the present study, enhanced the number of clefts formed (Figs 1–3, 5, middle and bottom rows). As for the control mid 12-day gland, the epithelial branching took place after the lobular enlargement, during a further 10 h of culture (Fig. 1, top row). However, when a gland at the same stage was exposed to 1000 rad of X-rays and then cultured under the same conditions, it formed clefts without being followed by any lobular expansion (Figs 1, 4, bottom row). This might be explained as the result of a relative reduction in the protruding activity of the lobular epithelium in contrast to the continued maturation or cytodifferentiation of the mesenchyme, possibly accompanied by an accumulation of collagen. Consistently, it has recently been shown that the mesenchymal cells of mouse embryonic submandibular glands at the same stages as used in the present study are able to contract collagen gel (Nogawa & Nakanishi, 1986).

Strands of evidence presented here can answer another question as to whether the narrow clefts deepen towards the lumen (at this stage no lumen formation was yet observed) or deepen by outgrowth of the adjacent epithelium towards the mesenchyme. As evident in Fig. 3 (bottom row), sites of the initial shallow clefts at 1 h of culture of gland treated with 1000 rad of X-rays became deep after 6 h of culture. The preliminary analysis of other epithelial contours on the photographs taken at appropriate intervals revealed that the deepening was made possible by the successive movement of sites of clefts towards the lumen sides. These observations also indicated that cell proliferation is not required either for the initiation of cleft formation or for the deepening of cleft when cultured *in vitro*.

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