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Cell deletion by apoptosis during regression of rat parotid sialadenosis

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Abstract Enlargement of the rat parotid salivary glands was induced by repeated administration of isoproterenol. Mean wet weights of the treated glands increased steadily to 240% of control values. Following withdrawal of the drug, quantitative histological techniques were used to investigate the balance between hypertrophy, hyperplasia and apoptosis. The volume occupied by acinar cells relative to the total gland volume together with cytoplasmic:nuclear area ratios as measures of hypertrophy increased during the early experimental period. Similarly, serous acinar cell mitotic counts increased, indicating that hyperplasia had occurred. Apoptosis was demonstrated at light microscopical level to be the main mechanism for cell deletion as the glands returned to normal size and weight. The results indicate that hypertrophy and hyperplasia of serous acinar cells contribute to isoproterenol-induced sialadenosis. The experimental animal model demonstrates that these proliferative changes are completed by 48 h and thereafter are balanced by apoptosis as the glands recover their normal size and weight.

Key words Isoproterenol · Apoptosis · Rat · Parotid · Sialadenosis

Introduction

Apoptosis is an active process that plays a fundamental regulatory function in the control of the overall size of a cell population, being complementary but opposite to cell proliferation [46]. A close relationship between these fundamental biological processes has been established in several organs and tissues, whereby an excess of cells created by treatment with mitogenic stimuli is reduced by apoptosis during subsequent regression of parenchymal hyperplasia [12]. Among the prominent structural changes characteristic of apoptosis are shrinkage of cell volume, morphological conservation of most cytoplasmic organelles, dilatation of cytoplasmic reticulum, and peripheral condensation of nuclear chromatin. In the last few years there have been dramatic developments in our understanding of the molecular events that initiate or abrogate apoptosis, and a common set of regulatory events seems likely [11, 13, 40, 45]. Certain rules appear to govern the process of apoptosis. Apoptosis, in most cases, will not proceed in the absence of RNA and protein synthesis, supporting the concept that apoptosis is an active, genetically controlled process [3]. The proto-oncogene *bcl*-2, when over-expressed, can protect cells from both normal and experimentally induced apoptosis, possibly by altering mitochondrial function [20]. However, more recent work [21, 22, 38] suggests that neither apoptosis nor the protective effect of *bcl*-2 depends on mitochondrial respiration. Thus, it may be said that the cell cycle and apoptosis share a common regulatory pathway that diverges at some point so that the cell either divides or dies. In rat salivary glands cellular hyperplasia can be induced by administration of isoproterenol, a sympathomimetic amine that acts on β-adrenergic receptors [26, 35, 36]. In this investigation, we have induced sialadenosis in the rat parotid gland by i.p. injection of isoproterenol and thereafter monitored the sequence of histological changes in recovering salivary glands. The processes of acinar cell hypertrophy, hyperplasia and apoptosis were chosen for qualitative and quantitative study.

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Materials and methods

Thirty two male Wistar rats were used. Each rat was 9–10 weeks old and weighed approximately 300 g. Throughout the course of the experiment they were fed a standard laboratory diet with water ad libitum. Twenty-four experimental rats received sterile isoproterenol solution (isoprenaline hydrochloride B.P.) i.p. at a concentration of 1 mg/ml. In order to induce chronic sialadenosis, the drug was administered on three separate occasions at 3-day intervals. Therefore, the following regimen was used: each animal received 6 mg on days 1 and 2, and 9 mg on days 5, 8 and 9, result-

ing in a total of 39 mg isoproterenol over a period of 9 days. Eight control rats received equivalent volumes of sterile saline administered during the same time course. Each rat was sacrificed by injection of 1 ml of euthatal (pentobarbitone sodium B.P; 200 mg/ml) i.p. Animals were sacrificed on the same day as the final injection of the drug was given (day 0 post-treatment) and thereafter on days 1, 2, 4, 6, 8, 10 and 13 post-treatment.

Following cardiac failure an incision was made in the central surface of the neck, and the parotid salivary glands were dissected free from connective tissue, cervical lymph nodes and extraorbital lacrimal glands before their wet weight was measured. The parotid glands from each animal were then fixed and prepared for light and/or electron microscopy.

Tissue preparation

Following fixation in 10% buffered formalin, tissue blocks were cut for embedding in paraffin wax, sectioned (5 μm) and stained with haematoxylin and eosin and periodic acid-Schiff (PAS). In addition, fresh tissue samples were diced and fixed in 4% gluteraldehyde in 0.1 M cacodylate buffer (pH 7.2) at a temperature of 4°C. Tissues were post-fixed in buffered 1% osmium tetroxide (pH 7.2), dehydrated and embedded in Araldite resin. Semi-thin sections were stained with toluidine blue and examined by light microscopy. Ultra-thin sections stained with uranyl acetate and lead citrate were examined in a Joel 100cx electron microscope.

Counting methods

The gland constituents to be counted at various stages in the study were acinar cells, acinar cytoplasm, acinar nuclei, mitotic cells, apoptotic cells and finally all other cell types taken as a group.

The histometric technique employed in the present investigation has been previously reported from our laboratory [1, 43] and by others [33]. Briefly, tissue sections were viewed under light microscopy using a Leitz-SM-LUX microscope with an objective of magnification ×40 and an ocular lens of magnification ×8, giving an overall magnification of ×320. This magnification allowed ready identification of the gland components to be counted with the aid of a Merz point-counting graticule in the ocular. The grid comprises 36 points and semi-circular testlines [23]. To obtain an estimate of the number of points to be counted in each gland, cumulative percentages [15] were calculated. By the time 1000 points had been reached in replicate counts, the cumulative plot for each tissue component was stable, showing the sample to be of sufficient size. The sections were then scanned systematically using the stage micrometer to determine movement in the vertical and horizontal planes until a minimum total of 1000 points per gland had been counted. After each count had been completed, the sum of the points overlying a particular gland constituent was expressed as a percentage of the total number of points counted. According to stereological methods [16, 17], this percentage gives an approximation of the volume proportion occupied by the constituent in the gland. In this way the proportional volume (or volume density) occupied by acinar cells was calculated [2]. In a similar fashion cytoplasmic and nuclear area counts for serous acinar cells were undertaken and their ratio used to give an estimate of cell hypertrophy. Mitotic and apoptotic indices were assessed in the same way. For regression analysis of the data, an SPSS – X release 2.1+PRIME (University of Dundee) linear regression program run on a Prime 9955 computer was used together with Student's *t* test to determine statistical analysis as appropriate.

Results

The histological and fine structural features of control and isoproterenol treated rat parotid glands exhibited the main characteristics previously reported [25, 30, 34, 47]. Administration of isoproterenol resulted in parotid gland enlargement, which was associated with increased mitotic counts and cytoplasmic:nuclear area ratios. The mean wet weight of the glands increased more than two-fold. Apoptosis accounted for the subsequent regression of parotid gland size and weight to normal values by 2 weeks after treatment. The following results, therefore, are based on observation and measurement of structural changes occurring in the post-treatment phase of the experiment (i.e. from day 0), which coincides with the period starting with the final injection of isoproterenol and first animal sacrifice and extending to the conclusion of the study (day 13).

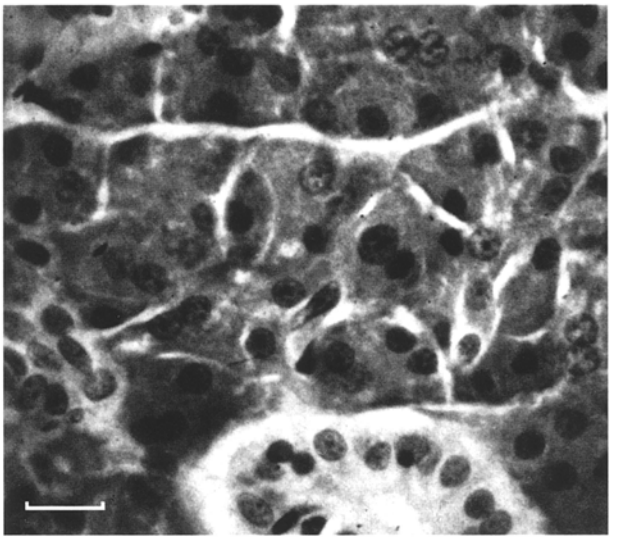

Fig. 1 Control parotid gland on day 1, showing closely packed serous acini. HE, ×40, *bar* 250 μm

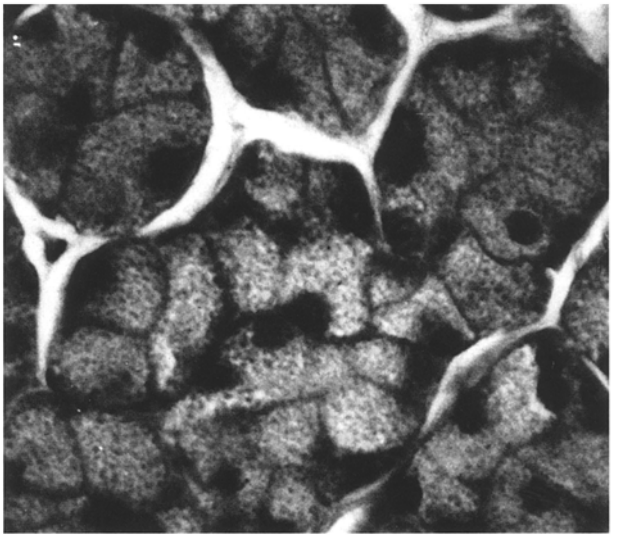

Fig. 2 Parotid gland of treated rat on day 1, showing hypertrophy of serous acinar cells with pale-staining (PAS-negative) secretory granules and prominent nuclei with scalloped margins. HE, ×40, *bar* 250 μm

Microscopy

Figure 1 shows that parotid gland lobules in control animals contained closely packed acini, which were pyramidal in shape with granular eosinophilic cytoplasm and basally oriented nuclei. Histological enlargement of serous acinar cells in treated animals was clearly observed and included hypertrophy of both nuclei and nucleoli. The nuclei appeared more granular than those in the corresponding cells in control glands (Fig. 2). The enlarged serous cells were packed with pale-staining (PAS–ve) secretory granules, which were closely packed in such a way that the nuclear margins were compressed, resulting in a scalloped appearance (Fig. 2). The nuclei themselves (which in normal serous cells occupy the basal third of the cell) abutted the basement membrane in the corresponding cells of treated glands. Serous cell outlines were also more distinct in treated than in control glands (Figs. 1, 2).

Mitotic figures were readily identified at an early stage in treated glands by their characteristic nuclear appearance (Fig. 3), but were absent from controls.

By day 2, apoptotic cells were observed. These were round or ovoid, in contrast to the surrounding pyramidal

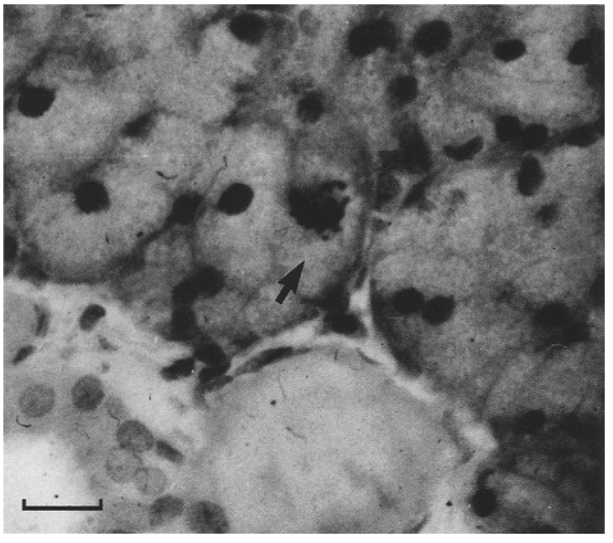

Fig. 3 Parotid gland of treated rat on day 2. Cell in mitosis (*arrow*). PAS, ×100, *bar* 100 μm

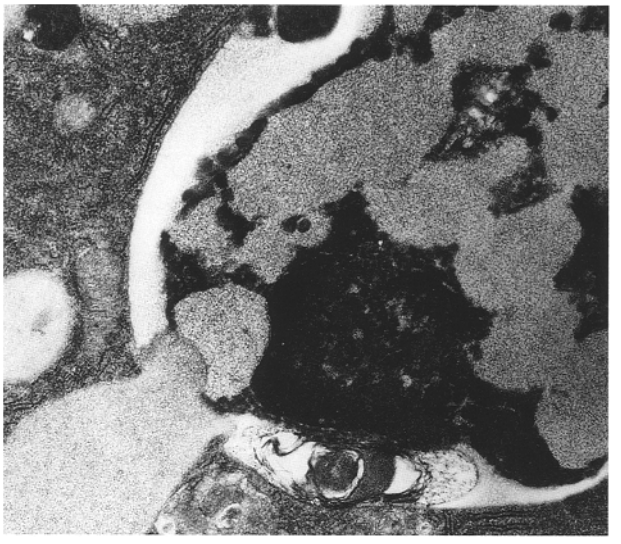

Fig. 5 Fine structural appearances of apoptosis in serous acinar cells from the parotid gland of a treated rat on day 4. Degeneration of nucleus and condensation of perinuclear cytoplasm are shown. ×6000

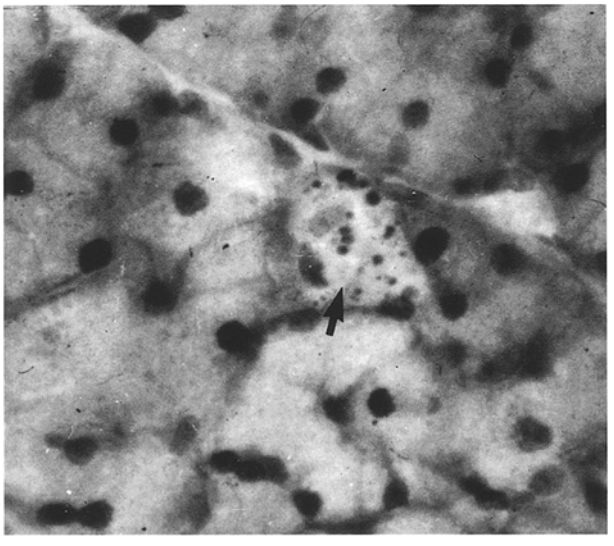

Fig. 4 Parotid gland of treated rat on day 4. Apoptotic body with pyknotic nuclear chromatin and clear halo (*arrow*). PAS, ×100, *bar* 100 μm

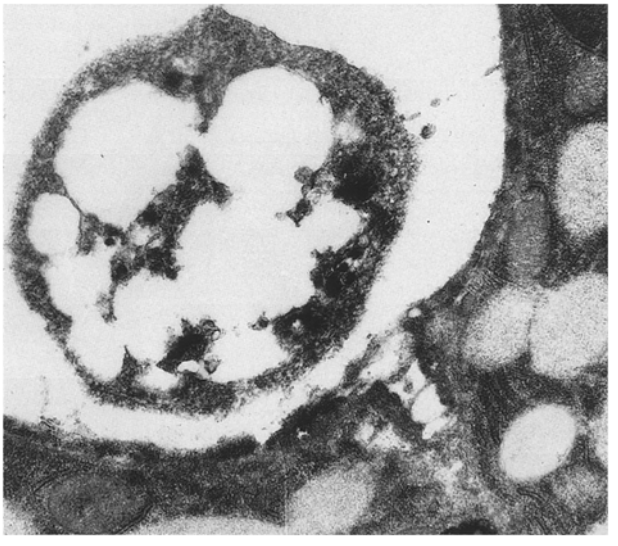

Fig. 6 Fine structural appearances of apoptosis in serous acinar cells from the parotid gland of a treated rat on day 6. Nuclear fragmentation, cytoplasmic degeneration and typical clear halo effect are shown. ×6000

cells, and showed pyknotic nuclear chromatin contained within a clear space or condensed eosinophilic cytoplasm (Fig. 4). In some cases their appearance suggested inward contraction of cytoplasm away from adjacent cells, leaving trailing threads of cytoplasmic material and membrane attached to them. Sometimes the basophilic fragments were absent and the apoptotic cells appeared simply as an ovoid or round clear zone with pale-staining cytoplasmic fragments. In PAS sections, apoptotic cells appeared as pale pink hyaline areas, enclosing dark red PAS+ve fragments (Fig. 4). The fine structural appearances of apoptosis were confirmed by ultrastructural examination of selected fresh material. Nuclear degeneration and cytoplasmic condensation are shown in Figs. 5 and 6.

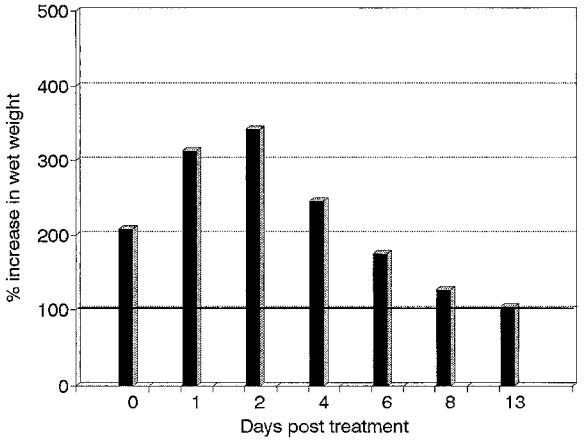

Fig. 7 Histogram showing the percentage increase in mean wet weights of parotid glands in treated over control rats during the 13 days following final administration of isoproterenol. Results are shown for treated rats as percentages of control (=100%)

Table 1 Mean proportional volumes (%) of acinar cells in parotid glands. On each day both parotid glands were assessed for 3 treated and 2 control animals; thus, for isoproterenol n=6/day and for controls n=4/day

Day	Isoproterenol (mean±SEM)	Control (mean±SEM)
0	87.1±0.59	75.4±0.89
1	85.9±0.87	72.1±1.15
2	93.2±0.72	80.2±0.55
13	78.4±1.01	76.5±0.78

Table 2 Mean cytoplasmic:nuclear area ratios in acinar cells in parotid glands in isoproterenol-treated and control rats. For treated group n=6/day and for controls n=4/day

Day	Isoproterenol (mean±SEM)	Control (mean±SEM)
0	7.49±0.82	6.18±0.43
1	11.65±1.11	3.47±0.27
2	11.34±1.30	3.20±0.37
13	3.95±0.64	3.76±0.54

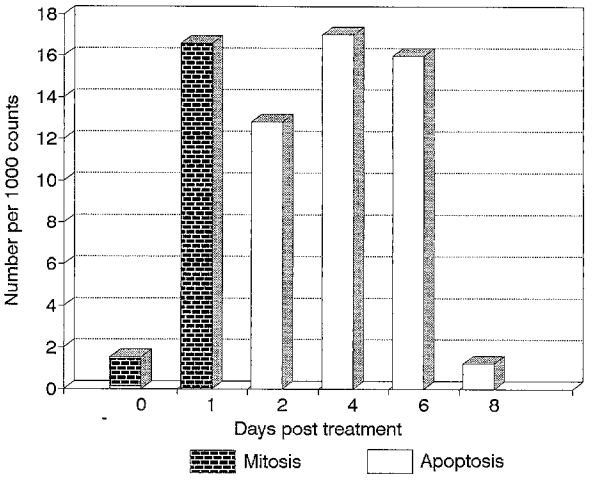

Fig. 8 Histogram showing mitotic cell count (*hatched*) and apoptotic cell count (*clear*), each per 1000 cells for parotid in days (0–8) following final dose of isoproterenol. After day 1, no mitotic count could be recorded. Apoptosis was noted from day 2–8. Neither mitosis nor apoptosis was observed in controls

Wet fixed weight of glands

The results for the wet fixed weight of glands are shown in Fig. 7 for day 0 to day 2. There was a significant increase (P<0.001) in weight in treated glands, to 240% of the corresponding control values. Thereafter, there was a significant decline (P<0.001) in the weight of treated glands through to day 13, by which time normal values obtained.

Histometry

The mean proportional volumes (%) of acinar cells in parotid glands in isoproterenol and control rats is shown in Table 1. By day 2 in treated animals, a significant increase (P<0.001) in acinar cell volume was noted. However, by day 13 the mean proportional volumes had returned to normal limits. From day 0 to day 2, significant differences were noted between control and treated animals (P<0.001).

Cytoplasmic:nuclear area ratios

The mean cytoplasmic:nuclear ratio for acinar cells in isoproterenol-treated and control rats is shown in Table 2. From day 0 to day 2 there was a significant increase (P<0.001) in ratios for treated animals. Thereafter the ratios fell back to normal by day 13. For control animals, the mean cytoplasmic:nuclear area ratio on day 0 was 6.18±0.43. A significant decrease was noted on day 1 and day 2. Significant differences between the ratios for treated and control animals were observed for the period day 1 to day 2, the magnitude of change being three-fold (P<0.001).

Mitotic and apoptotic counts

These counts from day 0 to the end of the experimental period are shown in Fig. 8. For mitosis on days 0 and 1 the mean values were 1.5 (±0.75) and 16.6 (±2.41) and for apoptosis the means were 12.8 (±3.20), 17.0 (±2.01), 16.0 (±1.62) and 1.2 (±0.40), respectively, for the remaining days of the experiment. Thus, in treated parotid glands the mitotic counts for serous acinar cells reached a sudden peak by day 1 after withdrawal of isoproterenol. An equally rapid decline was followed by waves of apoptosis over the next 6 days in serous acini in treated glands. Neither mitosis nor apoptosis was observed in the controls.

Discussion

In the present study, the high mitotic count in treated animals together with the absence of mitosis in controls clearly indicates that hyperplasia contributes to gland enlargement. It is known that isoproterenol stimulates DNA and RNA synthesis, which in turn is followed by an increase in mitotic activity, an activation of protein synthesis and accelerated cellular differentiation [8, 24, 27, 31]. The division of cells in advanced stages of differentiation is important to the growth of the developing rat parotid [29].

In the present study, the volume occupied by serous acinar cells relative to the total gland volume increased, as did the cytoplasmic:nuclear area ratios, clearly indicating that hypertrophy of these cells also contributes to gland enlargement following isoproterenol administration. It is interesting that on day 0 the cytoplasmic:nuclear area ratio for acinar cells in control animals was high, indicating a measure of hypertrophy. It is possible that the administration of isotonic saline may have lead to transient hyperhydration. Shannon and Chauncey [37] have shown that increased water intake in humans leads to hyperhydration and increased parotid salivary flow rates, suggesting a potential osmoregulatory function for salivary glands. Although hyperplasia and hypertrophy both contribute to gland enlargement, the precise timing of the former process during isoproterenol enlargement is far from settled [5, 7, 9, 32, 36, 39]. It seems that the number of cells (or clones of cells) induced to enter S phase and subsequently undergo mitosis is dose dependent. Barka [5] found that a single injection of isoproterenol (25 mg) significantly elevated DNA synthesis within rat submaxillary gland, as measured by labelled thymidine uptake, with maximum incorporation occurring 24 h after injection. Subsequently, Barka [6] has shown that RNA synthesis is also stimulated, with labelled uridine uptake reaching a peak at 18 h, and that DNA synthesis returns to normal levels with 48 h of the injection. Baserga and Heffler [8] have also noted that maximum mitotic activity occurs about 30 h after a single injection.

The occurrence of apoptosis during involution of isoproterenol-induced cell hyperplasia, as reported now by ourselves, appears not to have been reported before. However, morphological changes in rat salivary glands have been reported after actinomycin D administration [19], following a diet of liquid Metrecal [44] and during chronic ethionine treatment [14], and those described include "cytoplasmic condensations" (presumably apoptotic bodies). Atrophy of the parotid acinar cells following duct ligation appears to be the result of rapid apoptosis [41]. Recently, atrophy of other rat exocrine glands, such as the pancreas, has been shown to involve apoptosis following ethionine administration [42] and also in copper-induced deficiency [28].

We gained the subjective impression during counting that mitotic and apoptotic activity was not evenly distributed within the tissue but localised in fields, which suggests that clones of cells are undergoing apoptosis and mitosis. During the experiment proliferative changes were closely followed by apoptotic activity, the number of cells undergoing apoptosis being approximately the same as the number that had previously undergone mitosis. Similar bursts of mitosis were demonstrated during an investigation of the effects of isoproterenol on regenerating rat submandibular glands [10]. Apoptotic cells may be visible for only a few hours, for they are targets for immediate phagcytosis [45]. Improved identification may result from the recent reports of methods for in situ labelling of DNA strand breaks in apoptotic cell nuclei [4, 18].

The experimental model reported in this study seems to be a good one, since the processes of hyperplasia and cell deletion are clearly separated in time and appear to have a quantitative relationship in that mitosis is balanced by apoptosis. However, the means by which some individual cells are selected for deletion remains to be fully evaluated. This model of isoproterenol-induced salivary acinar cell apoptosis may prove a valuable system to examine biochemical and molecular events that trigger apoptosis and the nature of cell specificity.

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