Short- and Long-term Effects of Irradiation on Laryngeal Mucosa of the Rat

Mats Lidegran, Sture Forsgren, Åke Dahlqvist, Lars Franzén and Siw Domeij

From the Department of Otorhinolaryngology, Huddinge University Hospital, Karolinska Institute, Stockholm (M. Lidegran), the Departments of Anatomy (S. Forsgren), Otorhinolaryngology (Å. Dahlqvist), Oncology (L. Franzén) and Histology and Cell Biology (S. Domeij), University of Umeå, Umeå, Sweden

Correspondence to: Mats Lidegran, MD, Department of Otorhinolaryngology, Huddinge Hospital, S-141 86 Huddinge, Sweden. Tel: +46 8 58 58 74 77. Fax: +46 8 74 67 551. E-mail: Mats.Lidegran@klinvet.ki.se

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Although radiotherapy is often used to treat laryngeal carcinoma, there is little information on the effects of this treatment on laryngeal structures. Rats were irradiated to the head and neck region and the larynges were studied by light- and electron-microscopy and immunohistochemistry. Ten days after irradiation, a change in the ultrastructural appearance of the granules of the subglottic glands was observed. Substance P-, bombesin- and enkephalin-like immunoreactivity was increased in local ganglionic cells and glandular nerve fibres. The mast cells were reduced in number. At examination 4–6 months after irradiation, there were no obvious differences compared with controls concerning mast-cell numbers and neuropeptide expression. The ultrastructural changes seen in the subglottic glands remained to some extent. The results show that structural changes in the subglottic glands occur concomitantly with an increased expression of certain neuropeptides in the innervation of these glands, which implies a relationship between these two parameters. The mast cells respond drastically to irradiation, but in the long run, regeneration of these cells occurs.

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Radiotherapy is an important component in the treatment of laryngeal carcinoma and it is therefore important to elucidate the effects of this form of treatment on the structure and function of the normal larynx. Early changes in the irradiated larynx are the occurrence of hyperaemia and oedema in the mucosa and restricted laryngeal motion (1). Six months after single doses of irradiation to immature rabbit larynges, there is occurrence of squamous cell metaplasia and marked atrophy of the submucosal glands (2). In humans, late post-irradiation changes such as replacement of muscles and submucosal glands by dense collagen with dilated capillaries, cranial nerve damage and chondroradionecrosis have been described.

Recently, we observed that fractionated irradiation leads to an increase in the immunohistochemical expression of the neuropeptides substance P (SP), bombesin (BN) and enkephalin (ENK) in the innervation of the subglottic glands and in the ganglionic cells of the local laryngeal ganglia, as seen at examination 10 days after ceasing irradiation (3, 4). There is no information on whether the pattern of peptidergic innervation is the same long after irradiation. Changes in neuropeptide expression are interesting, as neuropeptides have neurotransmitter/neuromodulator effects, and because they act as growth hormones (5) and participate in repair mechanisms (6).

Irradiation has been found to exert a radical effect on the mucosal mast cells (MMCs) and connective tissue mast cells (CTMCs) in the gut, lung and salivary glands of the rat (7–9). It is not known whether the laryngeal MMCs and CTMCs are also affected by irradiation treatment, but knowledge of a presumed effect on the mast cells in the larynx would be of great relevance, as these cells are numerous in the larynx, including in man (10), and because these cells can release mediators known to participate in hypersensitivity reactions and inflammatory processes (11).

In the present study, the effects of fractionated irradiation on the rat larynx were examined, the questions to be answered being: (a) What overall morphological features can be seen in the short- and long-term after irradiation? (b) How does the pattern of the peptidergic innervation long after irradiation compare with the pattern seen in the initial stages? (c) What are the effects of radiotherapy on the MMCs and CTMCs? The larynges were examined by light- and electronmicroscopy and immunohistochemical means, 10 days and 4–6 months after concluding a course of irradiation. Attention was paid to the epiglottic and subglottic regions as these contain numerous mast cells and different types of epithelium and as both serous and mucous acini occur in the lamina propria of the subglottic region.

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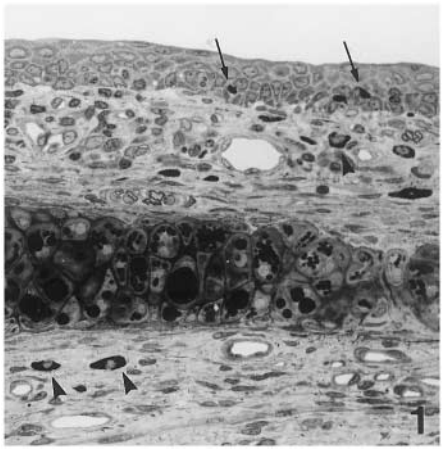

Fig. 1. Light micrograph of a specimen from the laryngeal side of the epiglottis of a control animal. MMCs (arrows) are visible in the epithelium, and CTMCs (arrowheads) in the lamina propria. × 260.

MATERIAL AND METHODS

Animals

Thirty-nine female Sprague-Dawley rats (200–250 g) were used. The animals had free access to water and pellets. Twenty-five animals were treated with irradiation to the head and neck region including the larynx. Fourteen rats served as controls and, apart from not being irradiated, were treated in the same way as the experimental animals, including being anaesthetized.

Irradiation

A medical linear accelerator, 6 MV (photons), was used to expose the animals to 6 or 8 Gy daily for 5 days, making a total dose of 30 or 40 Gy. During the irradiation, the rats were anaesthetized with Brietal® (Methohexital), 0.2 ml via a tail vein, and firmly held in prone position in a plastic mould. The irradiation was given from above. To deal with the build-up effect, a plastic plate 1.5 cm thick was placed on the mould. The absolute dosimetry was checked up in this way with an ionization chamber in a rat-like phantom and all scattering materials in the field were kept constant (12). Twenty-one rats, 13 of which were irradiated, were sacrificed after 10 days. Eighteen rats, 12 of which were irradiated, were examined after 4–6 months.

Tissue preparation for light- and electronmicroscopy

Sixteen animals anaesthetized with sodium pentobarbital (35–40 mg/kg i.p.) (9 irradiated, 5 of which were sacrificed after 10 days; 7 controls, 5 of which were examined 10 days after sham irradiation) were perfused for 5 min with 3% glutaraldehyde in 0.1 M cacodylate buffer. The larynges were then quickly excised, postfixed for 5–6 h in the same fixative, rinsed in 0.1 M cacodylate buffer overnight, thereafter fixed in 1% OsO_4 in the same buffer, dehydrated

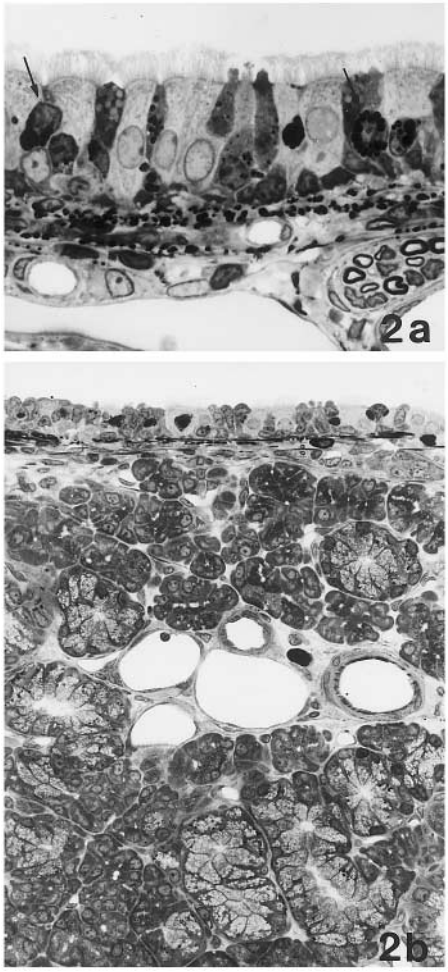

Fig. 2. Light micrographs of specimens from the subglottic region of a control rat, showing respiratory epithelium with ciliated cells and MMCs (arrows) (a). The lateral part contains numerous serous and mucous acini (b). a × 650, b × 260.

in a graded series of ethanol solutions and embedded in Poly/Bed (Polysciences Inc., Warrington, PA, USA). For light microscopy (LM), semithin (0.5–1.0 μm) sections were cut perpendicularly to the long axis of the larynges. The sections were stained with 1% toluidine blue for demonstration of tissue morphology and for identification of MMCs and CTMCs (13, 14). For electronmicroscopy (EM), ultrathin sections (about 70 nm), cut from the epiglottic and the subglottic regions, were collected on gold grids and contrasted with uranyl acetate and lead citrate and examined under a Jeol 100 CX electron microscope.

Calculation of number of CTMCs and MMCs

To determine the number of MMCs and CTMCs per mm^2, the total numbers of CTMCs and MMCs with a visible nucleus in the epi- and subglottic cross-sections of specimens processed for LM of the 16 rats referred to above were counted, and the mean number/mm^2 from each rat (three sections from each specimen) was calculated. As the

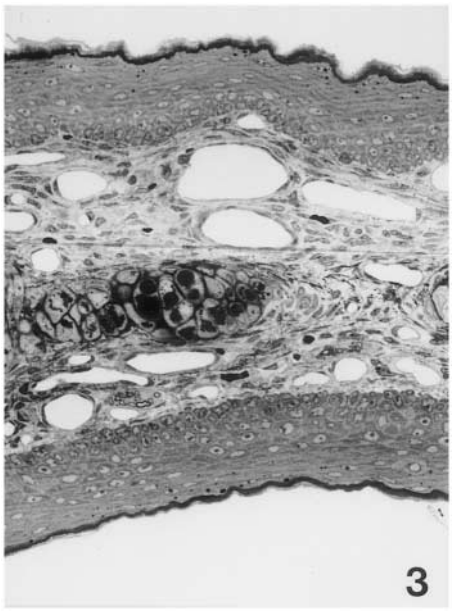

Fig. 3. Light micrograph from the epiglottis of an irradiated rat 10 days after irradiation. The epithelium is keratinized on both sides. No MMCs are visible in the epithelium. The blood vessels in the lamina propria are dilated. × 450.

lamina propria in the subglottic region consists largely of serous and mucous acini and ducts and few CTMCs, the mean number of CTMCs/section is presented as well (14).

To estimate the different areas, cross-sections from the epiglottic and subglottic regions were photographed in LM with a video camera (Cohu Inc., San Diego, CA, USA), and images were captured (Media Grabber, RasterOps Corp., Santa Clara, CA, USA) in a Macintosh IIsi computer. The grabbed images were transferred to a Power Macintosh 7100/80 and examined with image analysis software (IP lab Spectrum, Signal Analytics Corp., Vienna, VA, USA). In the epiglottis, the area of lamina propria (excluding the central cartilage) and the area of the laryngeal side of the epithelium were plotted manually in each section. In the subglottic region, the area of lamina propria inside the cartilage ring or musculature – including serous and mucous glands, ducts and connective tissue – was plotted manually. To measure the area of the subglottic epithelium, its height was measured with the same equipment at eight different measurement points in each section, after which the mean height was calculated. The length of the epithelium in every specimen was plotted on the screen.

Immunohistochemical procedures

From 23 anaesthetized animals (16 irradiated, 8 of which were sacrificed after 10 days; 7 controls, 3 of which were examined 10 days after sham irradiation) (sodium pentobarbital, 35–40 mg/kg i.p.), the larynges were excised, and the animals were killed by exsanguination. The larynges were fixed by immersion overnight at 4°C, in a solution of 4% formaldehyde in 0.1 M phosphate buffer, pH 7.0. The specimens were then washed overnight in Tyrode's solution containing 10% sucrose at 4°C. The larynges were cut transversely at points corresponding to the epiglottic and

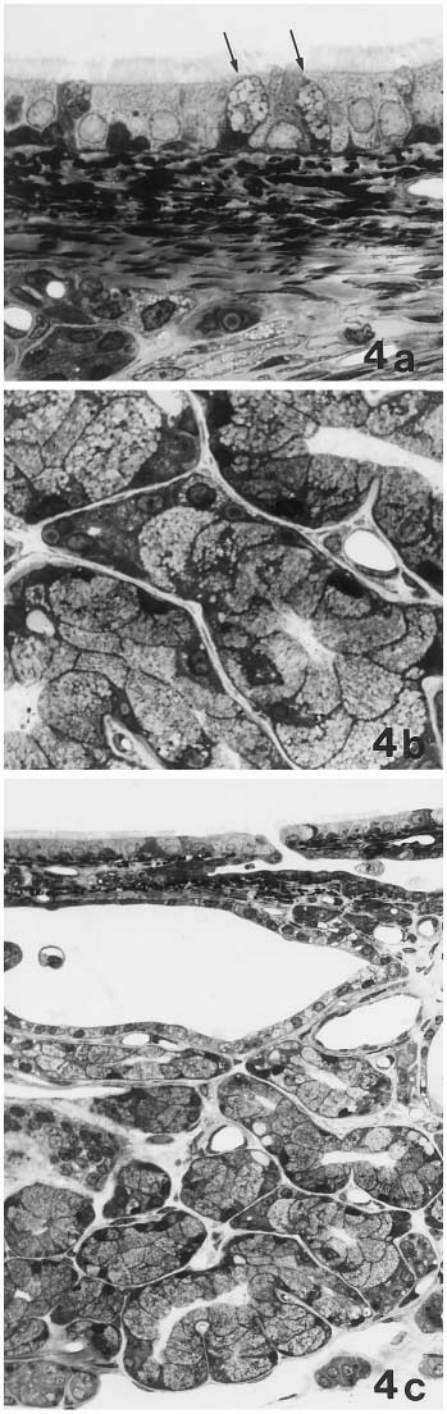

Fig. 4. Light micrographs of the subglottic region from an irradiated rat 10 days after irradiation, exhibiting an epithelium with several overfilled goblet cells (arrows), but no visible MMCs (a). In lamina propria, the mucous acini are seen to be filled with granules (b, c). a × 650, b × 650, c × 260.

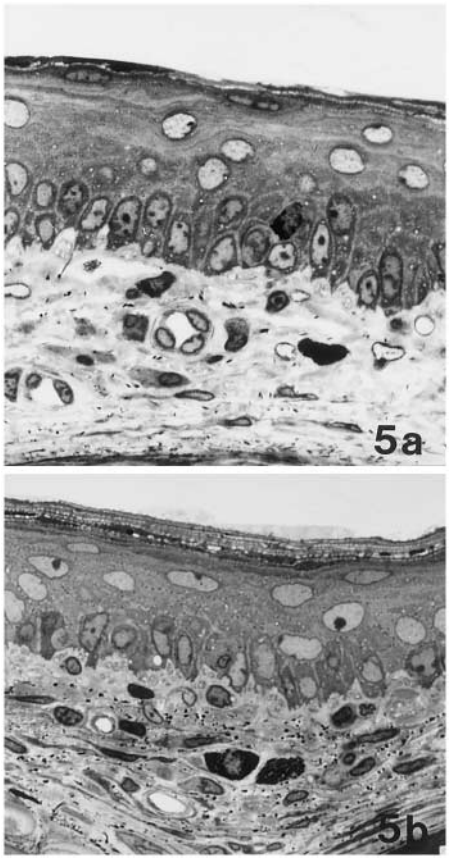

Fig. 5. Light micrographs of the laryngeal side of the epiglottic epithelium from a control rat (a) and a rat 4–6 months after irradiation (b). The control is age-matched. Although both display a keratinized epithelium, this is more marked in the irradiated rat. × 625.

subglottic regions. Thereafter, the specimens were mounted on thin cardboard in OCT embedding medium (Miles Laboratories, Naperville, IL) and frozen in propane chilled with liquid nitrogen. Specimens from controls and irradiated animals were frequently frozen together, enabling simultaneous processing of experimental and control tissues. Extensive series of 10 μm thick sections were cut, dried and processed for immunofluorescence or tissue morphology [NADH-tetrazolium reductase] (15).

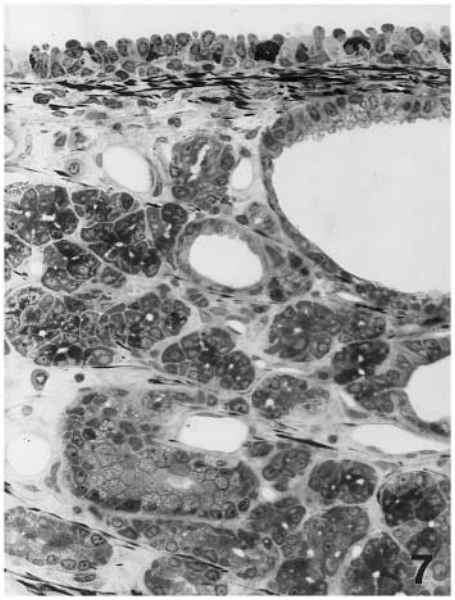

Fig. 7. Light micrograph from the subglottic region of an irradiated rat 4–6 months after irradiation. In the lamina propria, there are fewer acini and an increase in connective tissue. × 260.

The immunohistochemical procedures are described in detail elsewhere (16). The sections were incubated with a 1% solution of the detergent Triton X-100 (Kebo Lab., Stockholm) in phosphate-buffered saline (PBS), rinsed in PBS, pH 7.2, and incubated in normal swine serum in PBS. The sections were incubated with the primary antibody for 60 min at 37°C, washed in PBS, immersed in fluorescein-isothiocyanate-conjugated swine antirabbit IgG (Dakopatts, Denmark), diluted 1 : 40, for 30 min at 37°C, washed in PBS, and finally mounted in glycerol : PBS (1 : 1). The sections were examined in a Leitz Orthoplan photomicroscope equipped with epifluorescence optics.

Antibodies

Rabbit antibodies to SP (1 : 200, UCB, Brussels, Belgium), calcitonin gene-related peptide (CGRP) (1 : 100, Amersham Int., Buckinghamshire, UK), leu-enkephalin (ENK)

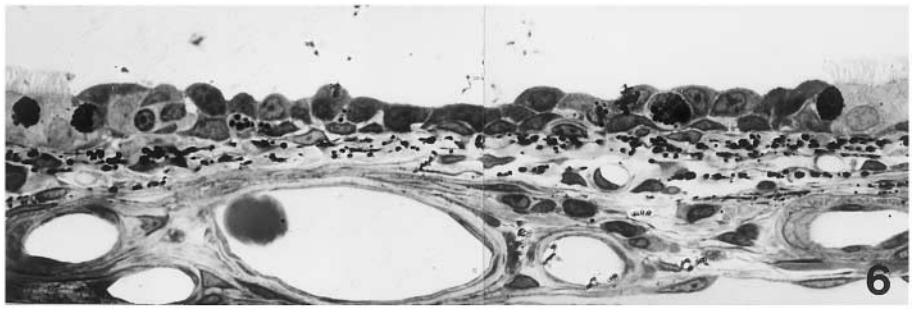

Fig. 6. Light micrographic montage from an irradiated rat 4–6 months after irradiation, showing metaplasia in the subglottic epithelium. × 450.

(1 : 100, Amersham), 5-hydroxytryptamine (5-HT) (1 : 200, Eugene Tech. Int. Inc., Allendale, NJ, USA), BN (1 : 40, Amersham), were used. The specificity of the antisera was tested by overnight incubation with corresponding antigen (10–20 μg) in 1 ml antiserum. As it is well known that BN antiserum cross-reacts to some extent with SP (4, 17), the BN-antiserum was always preabsorbed with SP (10–20 μg per ml) before incubation on the sections. The immunoreaction detected after this latter procedure is subsequently referred to as BN-like immunoreactivity (-LI). The leu-ENK antiserum shows partial cross-reactivity with met-ENK and minimally with beta-endorphin. We refer to the immunoreaction obtained after staining with leu-ENK antiserum as 'ENK-LI'.

RESULTS

The epiglottic and subglottic regions were examined with LM (Figs. 1–7), EM (Figs. 8–11) and by immunohistochemistry (Figs. 12–15). The examinations were carried out 10 days ('short-term') and 4–6 months ('long-term') after cessation of irradiation. There were no obvious differences in results between the rats given 30 or 40 Gy.

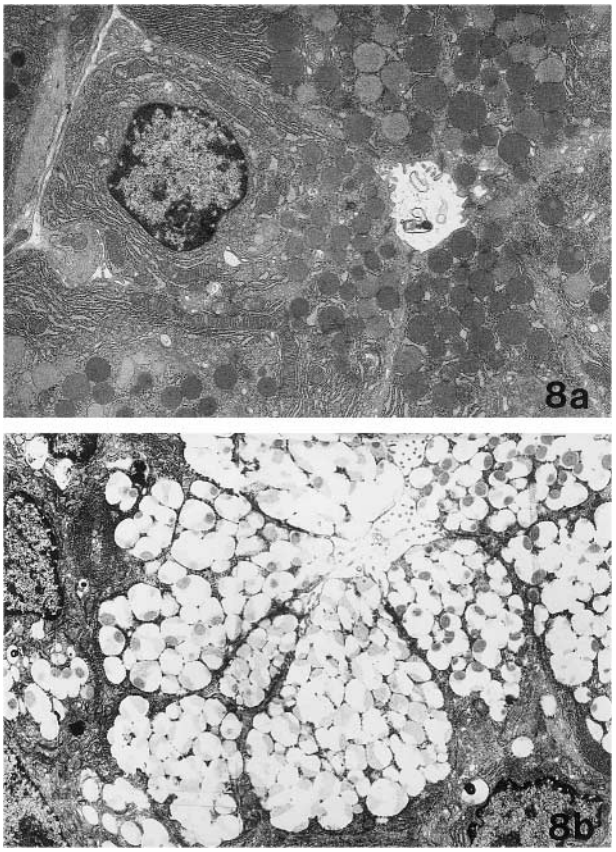

Fig. 8. Electron micrographs of a short-term control rat, showing some of the serous acini (a), and mucous acini (b). a × 4000, b × 2900.

Morphology, short-term effects

Control animals. LM showed the epiglottis to be covered with a stratified squamous epithelium, keratinized on the lingual side. In the epithelium, especially on the laryngeal side, MMCs were identified by their variously sized and orthochromatically stained granules (Fig. 1) (Table 1). Apart from cartilage and connective tissue, the lamina propria consisted of nerves, blood vessels and CTMCs. The CTMCs were recognized by their uniformly sized and meta- or orthochromatically stained granules, localized mainly on the laryngeal side (Fig. 1) (Table 2). In the subglottic region, there was a pseudostratified columnar epithelium, containing numerous MMCs (Fig. 2a) (Table 3), but also other granulated cells such as serous and goblet cells. In lamina propria, a small number of CTMCs were observed, being located mostly in the anterior and posterior parts of the larynx (Table 4). Serous and mucous acini were found especially in the lateral parts (Fig. 2b).

In EM, the acinar lumina were observed to be round, with visible villi. The serous acinar cells contained round, centrally located nuclei, and numerous round granules, electron-dense and of uniform density (Fig. 8a). In the mucous acinar cells, the nuclei were elongated and present in the basal part of the cells. The granules in these cells were electron-lucent, and bipartite, i.e. consisting of two components, a larger lucent part and a small denser core (Fig. 8b).

Irradiated animals. In LM, in both regions examined, scarcely any MMCs were found in the epithelium, and there were fewer CTMCs in lamina propria (Tables 1–4). The epithelium in the epiglottis was keratinized on both the lingual and the laryngeal side. The blood vessels in lamina propria were dilated (Fig. 3). In the subglottic region, the goblet cells were hypertrophied, compared with short-term controls. In other respects the epithelium appeared unaffected (Fig. 4a). In lamina propria, the mucous acini were hypertrophied, while the serous acini were difficult to detect, as the granules were not clearly identifiable (Fig. 4b, c).

In EM, in the epithelia of the two regions, no remnants of MMCs could be observed. The intercellular contacts were intact, no widened intercellular gaps being observed. In the subglottic region, several of the epithelial serous cells had few secretory granules compared with controls, those present being apically located. In lamina propria, a widened rough endoplasmic reticulum (RER) and lysozymes were more numerous in the glandular cells, compared with short-term controls (Figs. 9a, b). In the serous acinar cells, the granular structure was less electron-dense, but had small electron-dense areas, as compared with the controls (Figs. 9b, c). Mucous acinar cells were overburdened with granules varying considerably in electron density. The nuclei were pushed even further towards the base, and were compressed by granules (Fig. 9d).

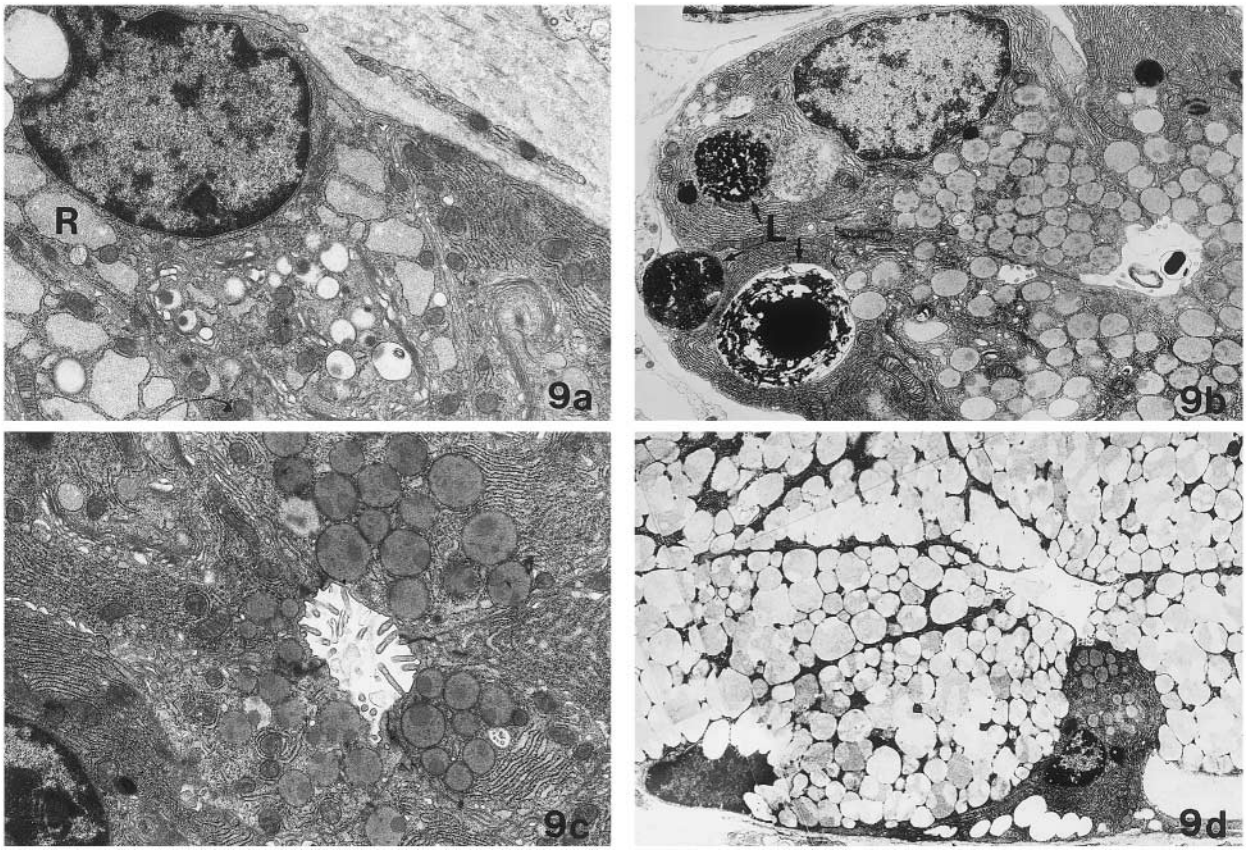

Fig. 9. Electron micrographs of an irradiated rat 10 days after irradiation. A serous acinar cell with a dilated RER (R) (a), and large lysozymes (L) were visible (b). The granular structure is less electron-dense and has a patchy appearance, with small, denser areas (c). In mucous acini, the dense core in the granules is less prominent, and the cell is overburdened with granules that deform and push the nucleus basewards (d). a × 6500, b × 5400, c × 5800, d × 3300.

Morphology, long-term effects

Control animals. In LM, in comparison to short-term controls, there was no obvious difference in the numbers of MMCs in the epithelium, or of CTMCs in lamina propria, in the two regions studied. The epiglottic epithelium was keratinized on both sides, i.e. also on the laryngeal side (Fig. 5a). No other morphological changes were found in the epithelium. In the subglottic region, there was an increased amount of connective tissue in between the glandular acini.

In EM, there were no obvious additional differences, compared with short-term controls (Fig. 10).

Irradiated animals. In LM, in the two regions examined, MMCs and CTMCs were again observed without any obvious differences in morphology or number, as compared with controls (Tables 1–4). The epiglottis showed a morphology similar to that seen in aged-matched control animals, though the epithelial keratinization was more pronounced (Fig. 5b). In the subglottic region, small areas of a low cuboidal or stratified squamous epithelium, i.e. metaplasia, were sometimes found (Fig. 6). In lamina propria, there was an increase in connective tissue and a reduced number of acini, as compared with both rats examined 10 days after irradiation and aged-matched controls (Fig. 7). Serous acini in particular were decreased in number.

At the EM level, in the subglottic region, the lumina of the serous acini were wide and irregular and had few villi.

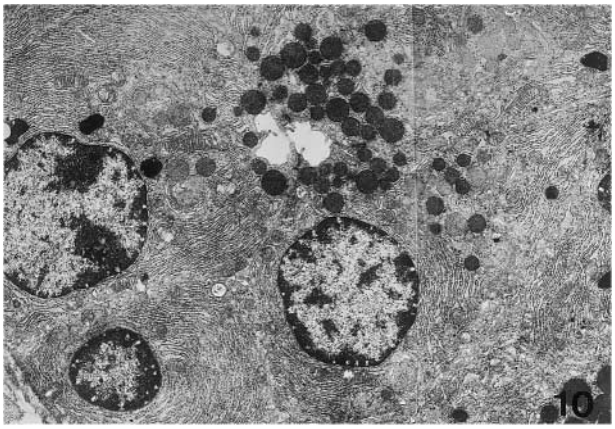

Fig. 10. Electron micrographic montage of a long-term control rat, showing electron-dense granules. × 3500.

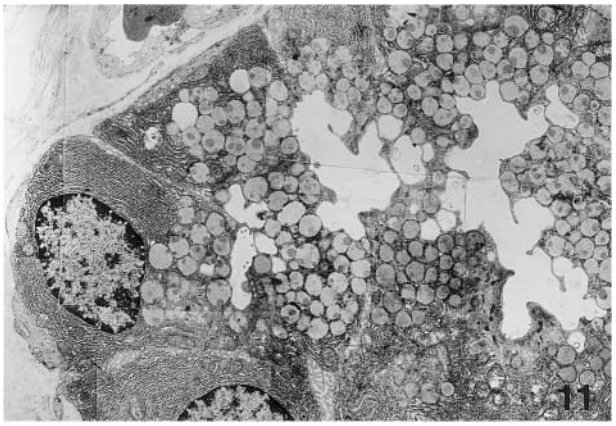

Fig. 11. Electronmicrographic montage of an irradiated rat 4–6 months after irradiation. The granules have a similar appearance to that in rats examined 10 days after irradiation. The lumen is wide and irregular, with few villi. × 3300.

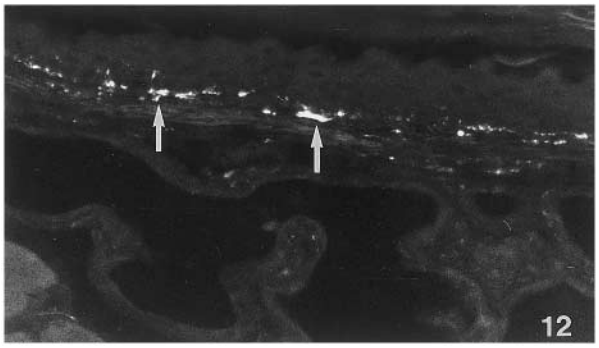

Fig. 12. A section, processed for SP, from the subglottic region of an irradiated animal examined 4–6 months after irradiation. Immunoreactive nerve fibres are observed basally in the epithelium (arrows). × 310.

The granules were less electron-dense than in the age-matched controls, but still exhibited small electron-dense areas, i.e. similar to those in irradiated rats examined after 10 days (Fig. 11). The mucous acini were rather similar in appearance, containing granule-filled cells, though not as enlarged as in rats examined 10 days after irradiation.

Immunohistochemistry – short- and long-term effects

Epithelium and lamina propria. The patterns of expression of SP, CGRP, BN and ENK were similar in both control groups and both groups of irradiated animals. This included the occurrence of a large number of nerve fibres showing SP- and CGRP-LI in the stratified squamous epithelium on the laryngeal side of the epiglottis, but fewer such fibres on the lingual side, and a moderate number of these fibres in the pseudostratified columnar epithelium in the subglottic region (Fig. 12). Some nerve fibres showing SP- and CGRP-LI were seen to be associated with the blood vessels in lamina propria. The distribution of fibres showing SP-LI resembled that of those showing CGRP-LI in the various regions. No nerve fibres were seen in the epithelium and lamina propria after processing for BN or ENK.

Glands. For all the peptides tested, the immunoreaction patterns in the subglottic glands of short- and long-term controls, and of irradiated animals examined 4–6 months after irradiation, were similar in appearance. This included the presence of a few fibres showing SP- or ENK-LI and only occasionally fibres showing CGRP-LI and BN-LI (Fig. 13a, c) in the glands. The number of fibres showing SP-, ENK- and BN-LI was markedly increased in the irradiated animals examined 10 days after irradiation (Fig. 13b) (cf. 3, 4). However, the level of expression of CGRP was the same as that observed for the other animals.

Ganglionic cells. Ganglionic cells occurred, either scattered or as clusters associated with vagal branches. These cells showed no immunoreactivity to any of the peptides tested in either type of control or in the animals examined 4–6 months after irradiation (Fig. 14b, c). On the other hand, parts of the ganglionic cells exhibited marked immunostaining after processing for SP (Fig. 14a), BN and ENK in animals examined 10 days after the end of irradiation (cf. 3, 4). CGRP-LI was not observed in ganglionic cells.

Cells showing 5-HT-LI. In the epiglottic epithelium, cells showing 5-HT-LI were not observed in any of the animals examined. In the subglottic epithelium of both types of

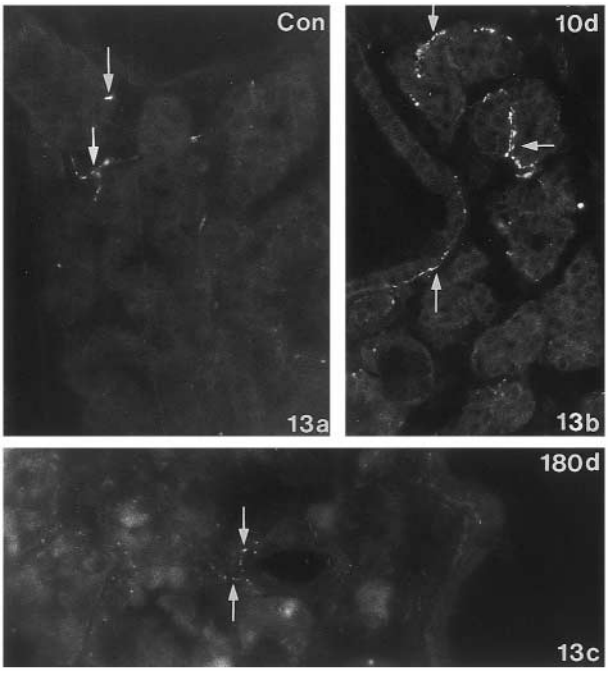

Fig. 13. Sections, processed for BN, of glands from the subglottic regions of a control animal (a), and animals examined 10 days (b) and 4–6 months (c) after irradiation. There are only a few faintly fluorescent nerve fibres in (a) and (c) (arrows), whereas there is a larger number of intensely fluorescent fibres in (b). Some of these are indicated (arrows). × 500.

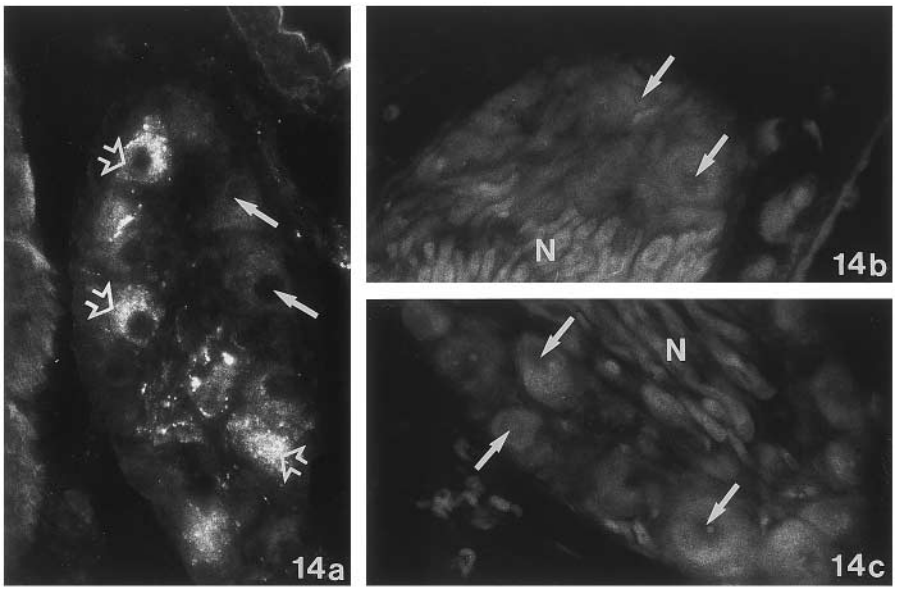

Fig. 14. Sections of small local ganglia of an animal examined 10 days after irradiation (a), from a control animal (b) and from an animal examined 4–6 months after irradiation (c). The sections were processed for SP. Open arrows indicate ganglionic cells exhibiting specific immunoreaction; ordinary arrows indicate cells exhibiting only autofluorescence. Note that none of the ganglionic cells in (b) and (c) show specific immunolabelling. N = nerve fascicle structure. × 500.

control animals, numerous cells showing 5-HT-LI were found (Fig. 15a), but not in irradiated rats examined after 10 days (Fig. 15b). In the rats sacrificed 4–6 months after the irradiation, 5-HT-LI cells were seen again in this region to the same extent as in controls. In the lamina propria, in both the epiglottic and subglottic regions, 5-HT-LI cells were found with a distribution consistent with that of CTMCs in the LM sections in controls as well as that of CTMCs in both groups of irradiated rats.

DISCUSSION

The present investigation, on the rat larynx, has demonstrated the effects of irradiation on its overall morphology and ultrastructure, on CTMCs and MMCs, and on neuropeptide expression. To a large extent these observations constitute new insights into irradiation-provoked effects on the larynx. As radiotherapy is frequently given for laryngeal carcinoma, it is important to know about any adverse effects this treatment might have on the laryngeal structures. By combining different methodologies, a comprehensive impression of the structural events that occur can be gained. The study has shown that numerous changes occur simultaneously 10 days after stopping the treatment: (i) structural changes in the subglottic glands, (ii) an increased expression of certain neuropeptides (SP, BN, ENK) in the innervation of these glands, and (iii) a noticeable disappearance of mast cells, particularly the MMCs. After 4–6 months, most of these changes were restored, i.e. the number of mast cells and the pattern of neuropeptide immunoreactivity in principle corresponded to that seen in controls. There were still ultrastructural changes in secretory granules. An important morphological observation at this stage was an increase in connective tissue in the lamina propria of the subglottic region. With the set-up for radiotherapy used here, it is a fact that other structures as well as the larynx had been irradiated. However, at least with regard to the patterns of neuropeptide expression, it seems that certain targets are affected. Thus, other structures encompassed by the irradiation in the present study as the nodose and superior cervical ganglia and spinal cord have previously been shown not to display any changes in the pattern of neuropeptide immunoreactivity (3, 18).

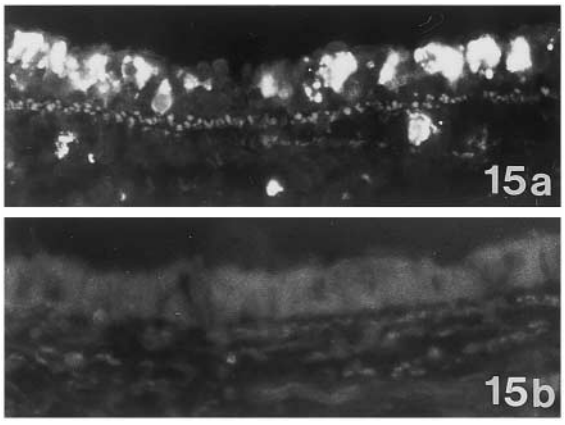

Fig. 15. Sections from the subglottic regions of a control animal (a), and an animal examined 10 days after irradiation (b). The sections were processed for 5-HT. There are several 5-HT immunoreactive cells in the epithelium and a few in the lamina propria in (a), whereas in (b), no immunoreactive cells are seen at all. × 310.

Table 1

Mean numbers (cells/mm^2) of MMCs in three sections of epithelium from the laryngeal side of the epiglottic region of rats examined 10 days (10 d) and 4–6 months (4–6 m) after irradiation and of controls

Controls (10 d) n = 5	Irradiated (10 d) n = 5	Irradiated (4–6 m) n = 4
1 090	0	576
276	11	153
303	0	434
549	0	20*
866	0	

* This rat had a thick keratinized layer on the laryngeal side of the epiglottis.

Short-term effects

The structural changes seen 10 days after the end of irradiation were confined to acini, both serous and mucous. The granules of the serous acini were pale and confluent, while the mucous acini were hypertrophied and overburdened with granules. At the EM level, the RER of the acini was more distended than in the controls. Furthermore, the granules in the serous cells were less electron-dense than those of controls, but contained small electron-dense areas, while in the mucous cells the granules closely abutted the nuclei. What is known in the literature on parotid and submandibular glands is that changes such as loss of glandular tissue integrity, oedema in serous cells with depletion of granules and vacuoles in the cytoplasm occur 3–4 days after irradiation in the rat (19, 20); 10–14 days after irradiation, a loss of serous cells and fibrotic changes take place (19). On the other hand, changes in granular structure, to some extent resembling those seen in the present study, have been observed in the parotid gland following treatment with beta-adrenoreceptor agonists. Sixty minutes after an injection of prenalterol, the acinar cells were almost devoid of secretory granules (21). After 2 weeks of daily injections, the granules had become less electron-dense, or had assumed a bipartite appearance caused by a mucoid transformation or a reorganization of the granular glycoproteins (22). In the rat submandibular gland, it has been shown that different proteins/glycoproteins predominate in electron-lucent/dense areas of the granules (23). This could indicate that the irradiation procedure leads to changes in the granular protein content.

Table 2

Mean numbers (cells/mm^2) of CTMCs in three sections of lamina propria of the epiglottic region of rats examined 10 days (10 d) and 4–6 months (4–6 m) after irradiation, and of controls

Controls (10 d) n = 5	Irradiated (10 d) n = 5	Irradiated (4–6 m) n = 4
104	40	109
105	41	67
72	34	122
111	22	51
116	47	

Table 3

Mean numbers (cells/mm^2) of MMCs in three sections of epithelium of the subglottic region, of rats examined 10 days (10 d) and 4–6 months (4–6 m) after irradiation, and of controls

Controls (10 d) n = 5	Irradiated (10 d) n = 5	Irradiated (4–6 m) n = 4
1 052	11	568
918	47	559
394	9	643
716	0	413
1 108	0	

As observed previously (3, 4), there was an increase in SP-, BN- and ENK-LI in the innervation of the subglottic glands 10 days after irradiation. A similar upregulation occurs in submandibular and parotid glands 10 days after irradiation (24, 25). Thus, the change in granular ultrastructure occurs simultaneously with the change in neuropeptide pattern, implying a functional relationship. The increase in the expression of certain neuropeptides in salivary gland tissue may be related to a reduced glandular secretion in response to irradiation, the neuropeptides being related to a compensatory stimulatory effect. Thus, SP and BN are well-known stimulants of airway mucus secretion (26, 27). The change in neuropeptide expression may also be related to a shift in the issuing of factors after the irradiation (cf. 28), as factors discharged from the target organ may influence neuropeptide expression in neurons (29). It is also a fact that the increased immunoreactivity seen 10 days after irradiation coincides in time with an increased regeneration activity of glandular cells in the parotid and submandibular glands, signifying irradiation-induced injury following a single 15 Gy dose (30).

Ten days after irradiation there was a decreased number (but no change in the morphology) of CTMCs in the laryngeal tissue. Consistent with this observation, a time-

Table 4

Mean numbers (cells/mm^2) of CTMCs in three sections in the lamina propria of the subglottic region of rats examined 10 days (10 d) and 4–6 months (4–6 m) after irradiation, and of controls. Numbers in parentheses are mean values (cells/section) of CTMCs from the three sections

Controls (10 d) n = 5	Irradiated (10 d) n = 5	Irradiated (4–6 m) n = 4
3.6 (7)	0.4 (0.6)	5.4 (8.7)
9.4 (15.7)	0.8 (1.3)	12.4 (19.3)
2.8 (2.8)	1.6 (2)	8.5 (14)
4.3 (8.7)	0.3 (0.3)	9.4 (13.3)
6.9 (17)	1.3 (1.3)	

dependent decrease in the number of CTMCs after irradiation has been observed in the main salivary glands in our laboratory, the decrease being evident 10 days after the end of irradiation (unpublished observations). MMCs, which are normally present in large numbers in the epiglottic and subglottic epithelia of the rat, were now scarcely detectable. In the gut mucosa too, MMCs disappear after irradiation without any increase in rat mast-cell protease II level, indicating that degranulation does not take place (9). For their development and growth, the MMCs are dependent on interleukin-3 (31), which is transcripted in T-lymphocytes (32). These latter cells are radiosensitive (33), which could thus explain the MMC depletion (9). Whatever the case, the decreased mast-cell number and the changes in the pattern of neuropeptide immunoreactivity were not regionally interrelated. The latter changes were observed in the nerve fibres associated with the acini, whereas MMCs are located in the epithelium and CTMCs chiefly in connective tissue in the anterior and posterior parts of the subglottic region.

Long-term effects

In the rats examined 4–6 months after irradiation, the serous granules were still less electron-dense than those of the controls, exhibiting small electron-dense areas. Thus, also at this stage the granules were not of normal appearance. An increased amount of connective tissue was observed in the subglottic lamina propria at the expense of the subglottic glandular structure. In the submandibular and parotid glands, a larger quantity of connective tissue and a reduced number of acini have also been observed long after irradiation (8). The effect is dose dependent (8, 25) and is more pronounced in the parotid gland, which contains only serous acini, which are more sensitive to irradiation than are mucous acini (8). As in the present study, the cells damaged by irradiation were replaced by connective tissue in those glands (8, 19).

In the subglottic epithelium, areas of metaplasia were sometimes seen as a long-term effect in irradiated rats. In the rat lung, a correlation has been shown between the irradiation dose and tumour incidence, with a threshold dose > 1 Gy, following inhalation of $^{239}PuO_2$ (Plutonium) in the range 0.01–62 Gy (34). Squamous metaplasia was not seen in controls, but in rats exposed to more than 1 Gy the incidence of metaplasia as well as tumour development increased with the inhaled dose (34).

Some 4–6 months after the end of irradiation, the pattern of neuropeptide immunoreactivity was similar in irradiated and control rats. Thus, it would seem that the initial triggering stimulus for neuropeptide up-regulation is no longer effective. Moreover, at this stage there was no obvious difference in the number of CTMCs, as compared with controls. In the lung, there is a marked increase in CTMCs at examination 4–8 weeks after irradiation, and a relationship exists between the increased number of CTMCs and an increase in connective tissue (7, 35). As suggested in the case of salivary glands (8), it seems that the CTMCs induce fibrosis in the lung, as treatment with the mast-cell degranulator compound 48/80 reduces the number of CTMCs as well as the extent of fibrosis in the latter organ (36). In the present study, the MMCs had returned in the epithelia of the epiglottic and subglottic regions after 4–6 months, and 5-HT-LI cells were again seen in the subglottic epithelium. This finding does indeed support the view that the 5-HT-LI cells in the subglottic epithelium correspond to MMCs (cf. 13, 14). In the intestine, a repopulation of MMCs begins 3 weeks after irradiation (9). Thus, it seems that MMCs in the intestine and the airways behave in a similar way in response to irradiation.

In conclusion, radiotherapy of the rat larynx leads to reduced numbers of MMCs and CTMCs and to increased SP-, BN- and ENK-LI expression 10 days after irradiation, changes that are reversed on examination 4–6 months later. The short-term neuropeptide changes in the tissue are restricted to the subglottic acini, in which granular changes are observed at the same stage. The simultaneous change in ultrastructure and neuropeptide expression implies a mutual relationship between these two phenomena. The most pronounced long-term effect is the increase in connective tissue in the subglottic region, with a reduced number of subglottic acini.

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