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Is radiation-induced degranulation of mast cells in salivary glands induced by substance P?

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Abstract

Although DNA is the critical target for the lethal effects of irradiation, the precise mechanisms by which irradiation causes damage in tissues and biological systems is not fully understood. In the present study, the number of mast cells and the expression of the neuropeptide substance P (SP) in salivary glands were examined 10 days after a regimen of irradiation. The irradiation was given as a single dose or 5 consecutive days with daily doses of 7 Gy up to a total dose of 35 Gy. In addition, the number of mast cells and the expression of SP were examined 2 and 24 h after a single dose of 7 Gy. Immunohistochemical staining for 5-hydroxytryptamine (5-HT) and staining with avidin peroxidase and toluidine blue were used to detect mast cells. At examination 2 and 24 h after irradiation treatment, no change in the number of mast cells and the pattern of SP expression was observed. Ten days after irradiation there was a remarkable reduction in the number of mast cells in all the three glands, but there was a marked increase in the number of nerve fibers showing SP-like immunoreactivity in the parenchyme. The results show that early time-dependent alterations in the density of mast cells occur in response to irradiation, and that these changes occur concomitantly with changes in the expression of SP. Since the peripheral nervous system is a main regulator of salivary gland function, it is tempting to speculate that the nervous system interacts with mast cells via SP in modulating irradiation provoked tissue responses in salivary glands.

Keywords: Irradiation; Neuropeptide; Substance P; Mast cell; Salivary glands

1. Introduction

Hampered salivary flow during the course of irradiation is an early sign of the radiosensitivity of salivary glands. In addition to direct influence on DNA, other cellular constituents such as cellular membranes also seem to be affected. Moreover, the observed time- and dose-dependent changes in the expression of growth factors, such as substance P (SP) and bombesin, have been suggested to be of importance in the modulation of the tissue reactions following irradiation [1,2]. The density of mast cells has also been shown to be affected in a radiation dose-dependent manner and these changes have been proposed to be related to late tissue obtained reactions, including fibrosis, in salivary glands [3] and lung tissue [4–7]. Mast cells contain a wide variety of inflammatory mediators such as histamine and are distributed within and/or near peripheral nerves in the tissue. In many tissues a morphological association between mast cells and neuropeptide-containing nerves has been demonstrated, favouring the occurrence of an inter-communication between the mast cells and these nerves [8–11]. Accordingly, neuropeptides, e.g. SP, can induce a release of histamine from mast cells in animals as well as in man [12–14].

In order to further delineate the effects of irradiation on peripheral tissues, we have evaluated the appearance of mast cells at early stages after this treatment, and examined whether the possible changes in mast cells are correlated to changes in the expression of SP in rat salivary glands. The results suggest that mast cells and SP-containing nerves might form a functional unit involved in the tissue response following irradiation in salivary glands.

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2. Materials and methods

2.1. Animals

White female 8-week-old albino rats of the Sprague-Dawley strain were used. They were fed on water and chow ad libitum and kept on a diurnal light schedule. Either 2 h, 24 h or 10 days following the last irradiation, the rats were fasted and were then killed, whereafter specimens were taken and processed for the analysis described later. The experimental procedure was approved by the animal ethic committee at the University of Umeå, and all efforts were made to minimise animal suffering.

2.2. Irradiation

Irradiation was carried out on a medical linear accelerator, 6 MV (dose rate 2.5 Gy/min), and was given on 5 consecutive days with daily doses of 7 Gy (35 Gy in total). Another group of animals was given a single dose of 7 Gy and analysed 2 or 24 h after irradiation. The irradiated volume included all major salivary glands. The rats were anaesthetized with Brietal (Methohexital) in the tail vein, fastened in a plastic mould in order to be firmly positioned during irradiation and were observed through a television camera. The absolute dosimetry was checked with an ionization chamber in a rat-like phantom and all scattering materials in the field were kept constant. Non-irradiated animals served as controls. The number of used animals are outlined in Table 1 and Fig. 9.

2.3. Tissue preparation

Irradiated and control rats were anaesthetized with sodium pentobarbital (40 mg kg^{-1} i.p.), whereafter all major salivary glands (parotid, submandibular and sublingual glands) were dissected out. The specimens were fixed by immersion overnight at 4°C in an ice-cold solution of 4% formaldehyde in 0.1 M phosphate buffer, pH 7.0. Thereafter the specimens were thoroughly washed in Tyrode's solution, containing 10% sucrose, at 4°C overnight. The specimens were mounted on thin cardboard in OCT embedding medium (Miles Laboratories, Naperville, IL) and frozen in propane chilled liquid nitrogen. Specimens of the glands from control animals were handled in parallel with specimens of corresponding glands from irradiated animals.

2.4. Sectioning

An extensive series of 7–8-μm thick sections were cut using a cryostat. The sections were mounted on slides pre-coated with chrome-alum gelatin, dried, and processed for immunofluorescence or stained for demonstration of NADH-TR activity [15] or stained with toluidine blue or avidin peroxidase [16].

2.5. Immunohistochemical procedures

The sections were incubated for 30 min in a 1% solution of detergent Triton X-100 (Kebo Lab, Stockholm) in 0.01 M phosphate-buffered saline (PBS), pH 7.2, containing 0.1% sodium azide as preservative, rinsed in PBS, and incubated in 5% normal swine serum in PBS supplemented with 0.1% bovine serum albumin (BSA) for 15 min. The sections were thereafter incubated with the primary antibody, diluted in PBS with BSA, in a humid environment. Incubation was performed for 60 min at 37°C. After incubation with specific antiserum against 5-hydroxytryptamine (5-HT) or SP and after washes in PBS, the sections were immersed in fluorescein-isothiocyanate-conjugated swine anti-rabbit IgG (Dakopatts, Denmark), diluted 1:40 v/v, for 30 min at 37°C in a moist chamber, washed in PBS, mounted in glycerol:PBS (1:1 v/v) and examined under a Leitz Orthoplan photomicroscope equipped with epi-fluorescence optics. (For further details refer to [1,2].)

In some sets of experiments, sections were processed for 5-HT, photographed, washed with PBS and thereafter stained with avidin peroxidase.

2.6. Antibodies

The 5-HT antiserum is a rabbit polyclonal antiserum raised against a serotonin-limulus hemocyanin conjugate (NT 102; Eugene Tech Int Inc., Allendale, NY).

Table 1
Mast cell numbers (per mm^2) in non-irradiated controls and at different time points following irradiation (mean ± SE)

	Control	2 h after 7 Gy	24 h after 7 Gy	10 days after 5×7 Gy
Parotid gland	25.2 ± 2.4 (n = 8)	28.6 ± 5.2 (n = 4)	35.5 ± 1.6 (n = 5)	5.7 ± 1.3* (n = 6)
Submandibular gland	27.9 ± 2.1 (n = 9)	29.0 ± 3.0 (n = 7)	29.0 ± 3.3 (n = 6)	6.3 ± 1.1* (n = 7)
Sublingual gland	27.9 ± 4.0 (n = 9)	19.9 ± 2.7 (n = 5)	26.9 ± 2.7 (n = 5)	6.4 ± 2.5* (n = 7)

Mann–Whitney test applied to group means.
*$P < 0.001$.

The working dilution of the antiserum was 1:100. Specific immunoreaction was not obtained in sections 132 incubated with 10–20 μg of 5-HT (Sigma, St Louis, MO) in 1 ml of antiserum. A rabbit antibody against SP (working dilution 1:200; code: i 675/002) was also used. The SP antiserum was purchased from UCB (Brussels). The specificity of the antiserum was tested by incubating control sections with antiserum preabsorbed with 10–20 μg of SP (Sigma) in 1 ml of antiserum.

2.7. Mast cell estimation

Mast cells were counted in 7-μm sections stained with avidin peroxidase under light microscopy, using a fixed field technique with a calibrated square 10x/25. At least 7 fields per section were counted and the results expressed as cells per square millimeter.

3. Results

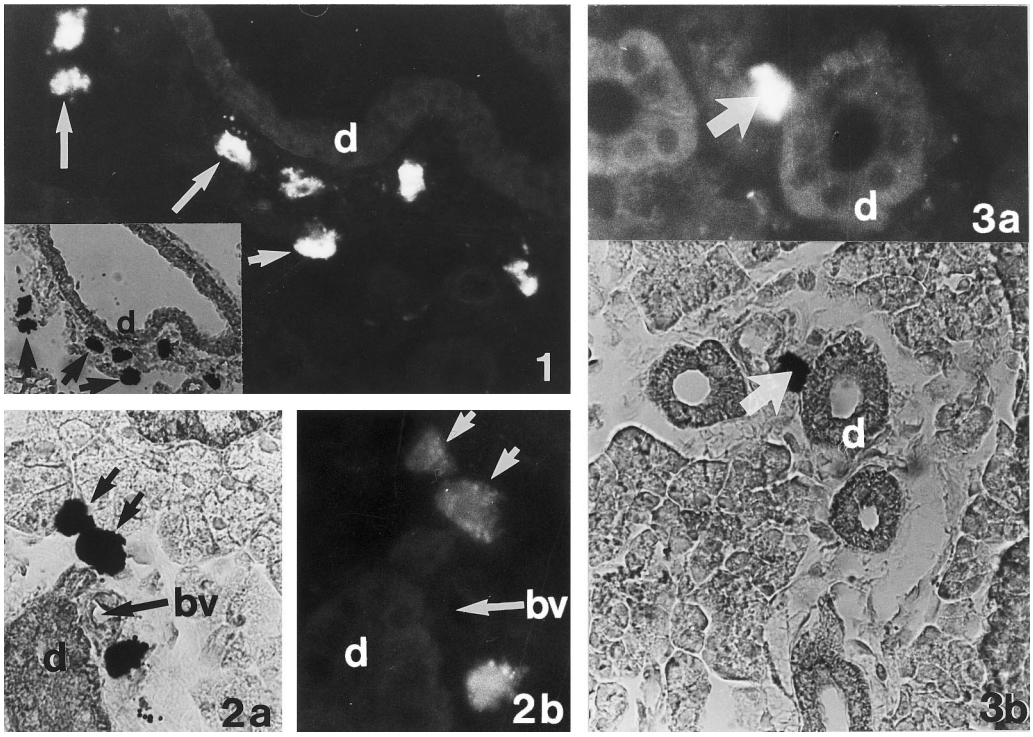
3.1. Mast cells

3.1.1. Controls

In the controls, mast cells were seen in all salivary glands after staining for 5-HT (Figs. 1–3). They were particularly located in connective tissue spaces, being associated with blood vessels and large ducts. In the parenchyme, mast cells were rare, however, a few cells were situated in association with the acini and small ducts (Fig. 3). A considerable number of positively stained cells were also observed in all three glands of the controls after staining for avidin peroxidase (Figs. 1–3). Examinations of sequentially double-stained sections showed that the avidin-positive cells corresponded to those that were stained with 5-HT-antiserum (Figs. 1–3). Also after staining with toluidine blue, cells showing a similar distribution as the avidin-positive cells were seen. However, the demarcation of the cells was better outlined after staining with avidin peroxidase.

3.1.2. Irradiated animals

At examination 10 days after a treatment of 35 Gy (7 Gy×5) there was a marked reduction in the number of 5-HT-immunoreactive cells (Table 1, Fig. 4), including all the various locations already referred to and all three salivary glands. In some connective tissue spaces where large ducts and blood vessels occurred, regions in which mast cells regularly occurred in the controls, no 5-HT-immunoreactive cells at all were observed (cf. Fig. 4). There was a parallel decrease in the number of stained cells after staining with avidin-peroxidase or toluidine blue (Table 1). Although sometimes also observed in the controls, mast cells displaying non-homogenous morphology and staining alteration with signs of extrusion of cytoplasmic granules from cells were regularly



Figs. 1–3. Sections of sublingual (Fig. 1), submandibular (Fig. 2) and parotid (Fig. 3) glands of control animals. All the sections were sequentially double-stained; staining for 5-HT (main Fig. 1, Fig. 2b and Fig. 3a) and for avidin (avidin staining shown in lower magnification). Mast cells are encountered after both types of staining and in all three glands. Some cells are marked with arrows. Note that there is an overlap between 5-HT-LI and positive staining for avidin. d, large ducts; bv, blood vessel. Fig. 1 ×310; inset Fig. 1 ×100; Fig. 2a and Fig. 3b ×310; Fig. 2b and Fig. 3a ×500.

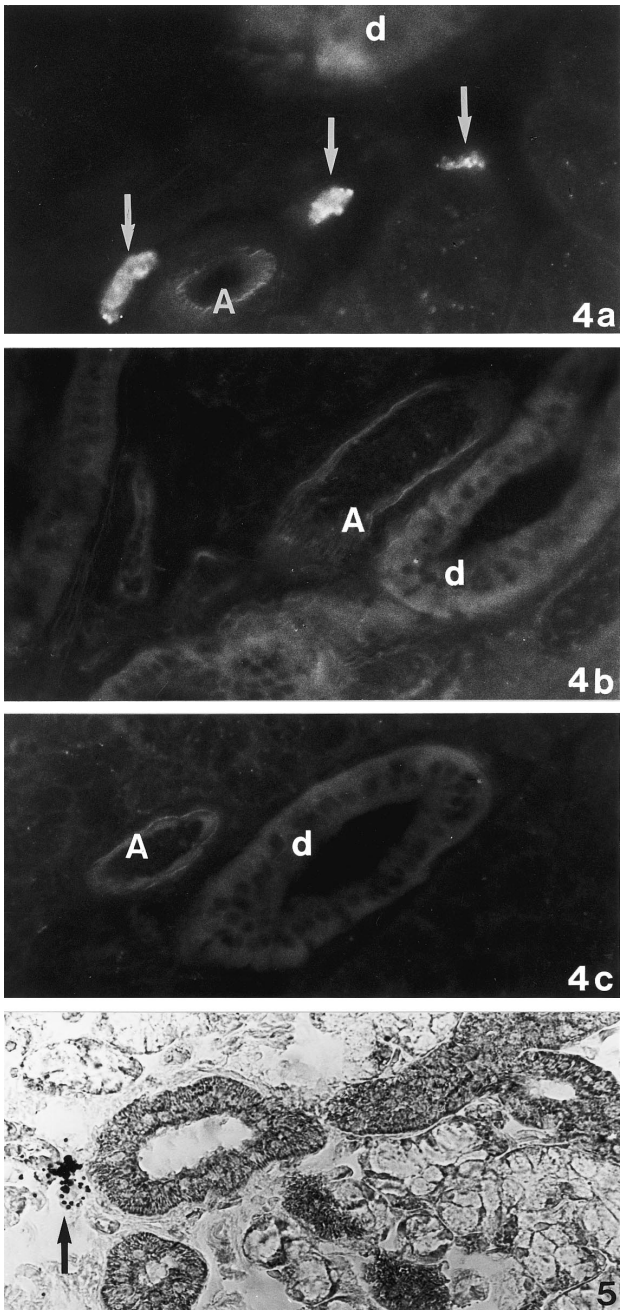


Fig. 4. Sections of submandibular glands of a control animal (a) and 10 days after an irradiation regime of 35 Gy (5×7 Gy) (b, c). Large ducts (d) and arterioles (A) occur in connective tissue spaces; only small parts of parenchyme are observed. Staining for 5-HT. Three mast cells are observed in (a) (arrows), none in (b) and (c). ×500.

Fig. 5. Section of the sublingual gland stained with avidin, 10 days after irradiation. A mast cell showing features of degranulation is seen (arrow). ×410.

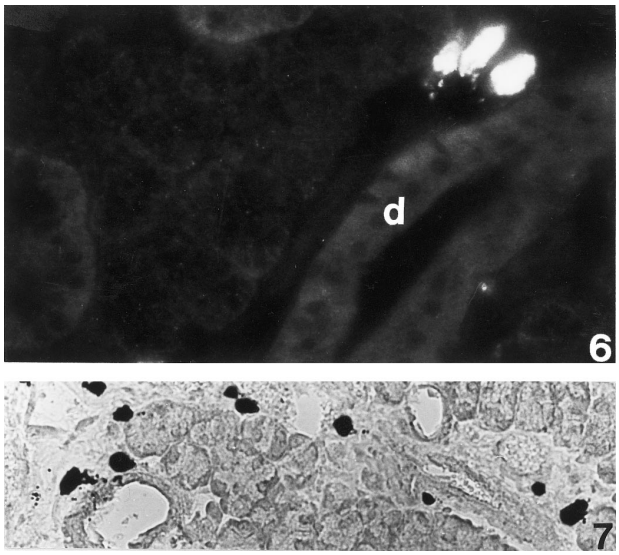
observed (Fig. 5), i.e. indicating degranulation of the mast cells 10 days after irradiation.

In order to evaluate whether irradiation causes a momentary degranulation of mast cells, the salivary glands were also examined 2 and 24 h after single dose of 7 Gy. In these occasions, the pattern of distribution as well as the number of 5-HT-positive and avidin-positive cells in all three major salivary glands appeared similar to that seen in the controls (Figs. 6 and 7; Table 1).

3.2. Substance P

3.2.1. Controls

A few nerve fibers displaying SP-like immunoreactivity (LI) were seen in close association with the acini and small ducts in all three major salivary glands of the controls. There were also regularly nerve fibers showing SP-LI in association with the large ducts and sometimes such fibers occurred close to the blood



Figs. 6–7. Sections of the submandibular gland (Fig. 6) and the sublingual gland (Fig. 7) processed for 5-HT (Fig. 6) and stained with avidin (Fig. 7), 24 h after irradiation (single dose 7 Gy). Mast cells occur in characteristic locations; close to large ducts and in connective tissue spaces. Fig. 6 ×500; Fig. 7 ×200.

vessels. In these latter locations, fibers showing SP-LI were also found grouped into nerve fascicles.

3.2.2. Irradiated animals

At 2 and 24 h after irradiation with 7 Gy, there were no obvious changes in the distribution and number of nerve fibers exhibiting SP-LI as compared to controls. Thus, a few nerve fibers were seen in the parenchyme (Fig. 8b, cf. Fig. 8a) and fibers were detected in association with large ducts (Fig. 8c) and blood vessels. Ten days after cessation of irradiation there was a marked increase in the number of immunoreactive fibers and in the intensity of SP immunolabelling in the fibers of the parenchyme of the glands (Fig. 8d). On the other hand, no detectable time-related changes were seen with regard to the SP-expression in association with main excretory ducts and blood vessels.

A schematic illustration displaying a comparative time-schedule of the changes in mast cells and SP-immunoreactive nerve fibers is shown in Fig. 9. As can be seen, the decrease in the number of mast cells at examination 10 days after irradiation is accompanied by an increase in SP-expression as determined via semi-quantitative analysis.

4. Discussion

The detailed mechanisms which can explain the pronounced radiosensitivity of tissues like salivary glands remain to be elucidated. Previously, mast cells with their potent mediators of inflammatory processes [3], and growth factors/neuropeptides [1] have in separate studies been proposed to interact with and modulate the radiation-induced tissue reactions. The results of the present study, showing a concomitant decrease in the number of detectable granule-containing mast cells and an increase in SP immunoreactive nerve fibers, might suggest that irradiation can induce degranulation of mast cells. SP is known to cause release of histamine from mast cells [12,13]. Although the upregulation of SP that occurs in salivary glands in response to irradiation is confined to parasympathetic innervation of the parenchyma [2] and mast cells most frequently are located in connective tissue spaces, the occurrence of an interaction between SP and the mast cells cannot be excluded. Neuropeptides are likely to be transported over a long distance and still be able to elicit activity [11].

In a previous study, we demonstrated radiation dose-dependent changes with regard to late obtained effects 180 days after irradiation, including an increase in the number of mast cells [3]. From the present study, it seems that irradiation had caused a marked degranulation of mast cells or at least a decrease in the number of detectable mast cells at 10 days following irradiation in all three glands examined. A degranulation of mast cells following irradiation has previously been suggested to occur in rat intestinal mucosa, with a return to basal levels after 20 days [17]. These results are thus in accordance with our study, the studies showing that irradiation may lead to mast cell degranulation at examination 1–2 weeks following irradiation.

The present results obviously exclude the occurrence of a direct effect of irradiation on mast cells and a subsequent degranulation. Thus, a single radiation-dose did not change the number of mast cells at examination 2 or 24 h after irradiation. Hence, degranulation of mast cells does not seem to occur at the very early stage following

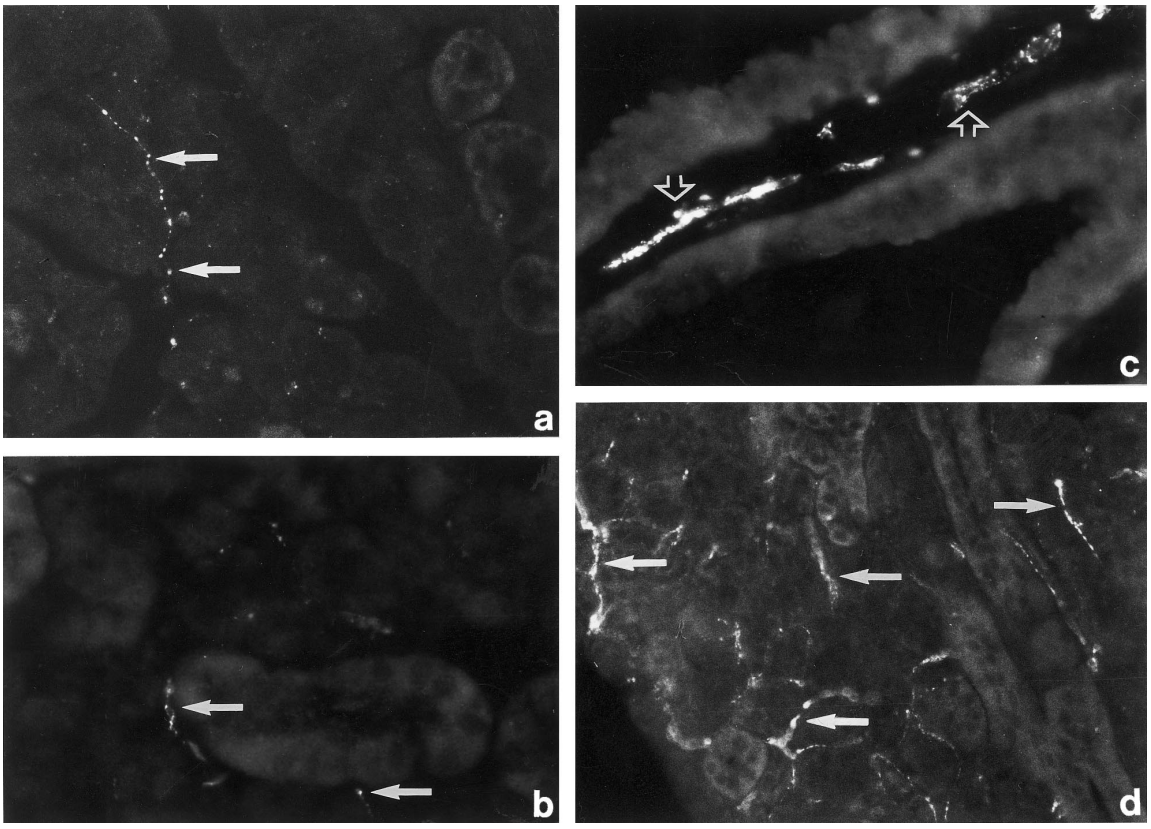


Fig. 8. Sections of submandibular glands processed for SP. Control animal (a), 2 h after an irradiation regime of 7 Gy (b, c) and 10 days after 35 Gy irradiation (d). There are a few varicose nerve fibers in the parenchyme in (a) and (b), whereas there is a large number of such fibers in (d) (filled arrows). In (c), nerve fibers in association with a large duct are seen (open arrows). Fig. 8a, c, d ×310; Fig. 8b ×500.

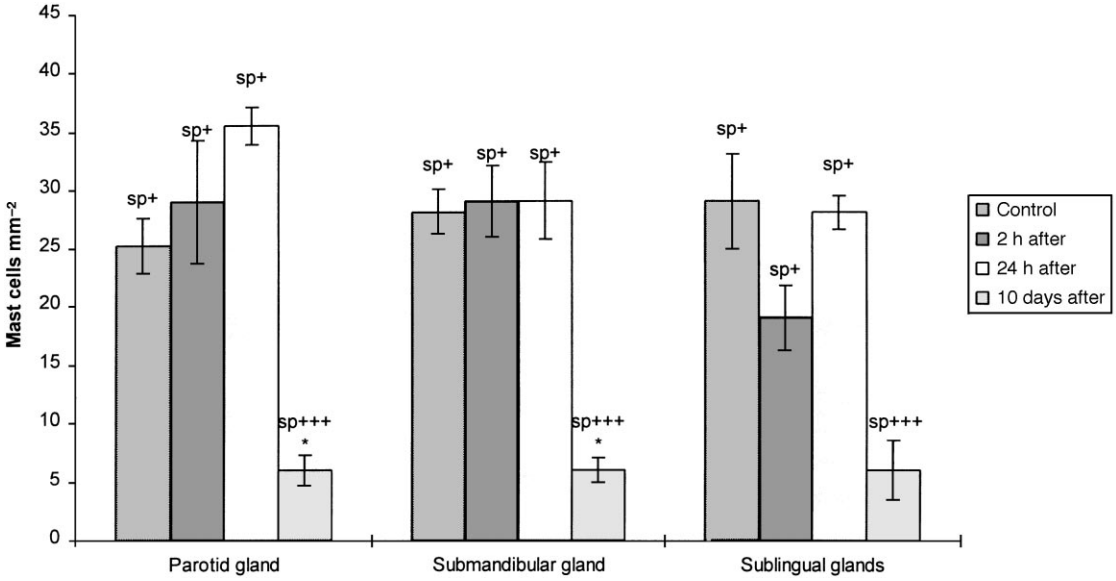


Fig. 9. Illustration of the relationship between quantitative light microscopical analysis of mast cells and semiquantitative analysis of SP-immunoreactive nerve fibers in the parenchyme of the glands of non-irradiated controls at different time points following irradiation. The analysis include parotid, submandibular and sublingual glands. The mast cell values are expressed as frequency of mast cells per mm^2. Error bars indicate standard error. *Mann–Whitney test resulted in P-values < 0.001, as compared to respectively non-irradiated control gland. The number of nerve fibers at each time point is expressed semiquantitatively, and shown in relation to time-matching mast cell values. sp+, a few nerve fibers showing SP-LI in the parenchyma; sp+++, a large number of SP-LI nerve fibers in the parenchyma. The number of animals in each group can be seen in Table 1.

irradiation. Interestingly, at the same stage as the decrease in mast cells occurs, there is an enhancement in the expression of SP (cf. also [1]). In this context, it is of interest to recall the observation already referred to that SP can induce histamine release from mast cells [12]. Also stimulation of trigeminal sensory fibers in rats containing SP induces morphological changes which reflect secretion from mast cells in dura mater [8]. Furthermore, SP-related peptides in the rat submandibular gland and oral mucosa have been proposed to stimulate histamine release [14,18]. In the present study, the fact that irradiation of salivary glands resulted in time-related changes in both the number of mast cells and the expression of SP in the same tissue, suggests that an inter-communication between mast cells and SP occurs. Moreover, release of substances such as histamine, serotonin and prostanoids from mast cells excites sensory nerve fibers to release SP from nerve terminals, causing further mediator release from adjacent mast cells [19]. Thus, there is a possibility of a bidirectional communication between mast cells and sensory nerves, leading to a mutual modulation in the final tissue response to irradiation. The observations that there were no detectable changes in mast cells and SP expression within days after irradiation, suggest that these events are not directly involved in radiation-induced apoptotic cell death in salivary glands, which has been shown to occur already a few days after exposure [20. However, the potential role of SP in other aspects of radiation sialoadenitis cannot be excluded [22].

Mast cells with their functional heterogeneity are distributed throughout the body, mainly located within or near peripheral nerves [23]. Two separate types of mast cells, mucosal mast cells and connective tissue mast cells (CTMC) are known. They display differences with respect to histochemical staining and biochemical and functional characteristics [24]. The mast cells observed in salivary glands are mainly of the CTMC type. The mast cells contain and release a wide variety of inflammatory mediators, which are stored in the granules and subsequently released into the extracellular space upon stimulation. The secretory constituents of mast cells, such as histamine, serotonin and prostaglandin secreted into the extracellular space cause oedema by a vascular permeability increase, and induces tissue destruction and cellular recruitment [23,25]. Moreover, the motor and secretory functions in the digestive tract are also affected by histamine [26]. Mast cells have also been connected to hyaluronic acid and collagen synthesis and to radiation-induced fibrosis in the lung [5–7,27] and the heart [28]. Therefore, it is plausible to propose that substances released from mast cells, induced by SP, for example, might be involved in the local regulation of reactions following radiation-induced inflammatory processes.

In conclusion, the time schedule for the decrease in number of detectable mast cells was found to correlate with a concomitant increase in the expression of SP in salivary glands. Therefore, we would like to propose that the nervous system is involved in the modulation of radiation-provoked tissue responses and it could also be speculated that degranulation of mast cells is of importance in tissue reactions following irradiation.

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References

[1] Aalto Y, Forsgren S, Kjörell U, Franzén L, Gustafsson H, Henriksson R. Time and dose related changes in the expression of substance P in salivary glands in response to fractionated irradiation. International Journal of Radiation Oncology Biology Physics 1995;33:279–305.

[2] Forsgren S, Franzén L, Funegård U, Gustafsson H, Henriksson R. Bilateral irradiation of head and neck induces and enhanced expression of substance P in the parasympathetic innervation of the submandibular gland. Neuroscience 1992;46(1):233–240.

[3] Henriksson R, Fröjd Ö., Gustafsson H, et al. Increase in mast cells and hyaluronic acid correlates to radiation-induced damage and loss of serous acinar cells in salivary glands: the parotid and submandibular glands differ in radiation sensitivity. British Journal of Cancer 1994;69:320–326.

[4] Hatamochi A, Fujiwara K, Uekin H. Effects of histamine on collagen synthesis by cultured fibroblasts derived from guinea pig skin. Archives of Dermatology Research 1985;277:60–64.

[5] Aldenborg F, Nilsson K, Jarlshammar B, Bjermer L, Enerbäck L. Mast cells and biogenic amines in radiation-induced pulmonary fibrosis. American Journal of Respiratory Cell Molecular Biology 1993;8:112–117.

[6] Nilsson K, Henriksson R, Hellström S, Tengblad A, Bjermer L. Hyluronan reflects of the pre-fibrotic inflammation in irradiation rat lung: concomitant analysis of parechyal tissues and bronchoalveolar lavage. International Journal of Radiation Biology 1990;58:519–530.

[7] Bjermer L, Franzén L, Littbrand B, Nilsson K, Ångström T, Henriksson R. Effects of smoking and irradiated volume on inflammatory response in the lung of irradiated breast cancer patients evaluated with bronchoalveolar lavage. Cancer Research 1990;50:2027–2030.

[8] Dimitradou V, Buzzi MG, Moskowitz MA, Theoharides TC. Trigeminal sensory fiber stimulation induces morphological changes reflecting secretion in rat dura mater mast cells. Neuroscience 1991;44(1):97–112.

[9] Stead RH, Dixon MF, Branwell NH, Riddell RH, Bienenstock J. Mast cells are closely apposed to nerves in the human gastrointestinal mucosa. Gastroenterology 1989;97:575–585.

[10] Domeij S, Carlsöö B, Dahlqvist Å., Forsgren S. Occurrence of mast cells in relation to the distribution of nerve fibers in the rat larynx. Acta Otolaryngology (Stockholm) 1991;111:981–989.

[11] Baraniuk JN, Kowalski ML, Kaliner MA. Relationships between permeable vessels, nerves and mast cells in rat cutaneous neurogenic inflammation. Journal of Applied Physiology 1990;68:2305–2311.

[12] Pauwels R, Joos G, Kips J, Van Der Straaten M. Interactions between neurotransmitters and inflammatory cells in the airways. International Archives of Allergy and Applied Immunology 1989;88:68–69.
[13] Lembeck, F., Gamse, R. Substance P in peripheral sensory processes. In: Porter, R., O'Connor, R. editors. Substance P in the Nervous System (Ciba Foundation Symposium 1991). Pitman, London, p. 35–54.
[14] Fazekas A, Györfi A, Irmes F, Rosivall L. Effect of substance P administration on vascular permeability in the rat dental pulp and submandibular gland. Proceedings of the Finnish Dental Society 1992;88(1):481–486.
[15] Dubowitz, V and Brooke, HM, Muscle Biopsy: a Modern Approach. WB Saunders, London, 1973, p. 20–23.
[16] Fritz P, Müller J, Reiser H, et al. Avidin-peroxidase. A new mast cell staining method. Acta Histochemistry 1986;(Supplement):235–239.
[17] Cummins AG, Munro GH, Huntley JF, Miller HRP, Ferguson A. Separate effects of irradiation and of graft-versus-host reaction on rat mucosal mast cells. Gut 1989;30:355–360.
[18] Györfi A, Fazekas A, Irmes F, Rosivall L. Effects substance P administration on vascular permeability in the rat oral mucosa and sublingual gland. Journal of Periodontology Research 1995;30:181–185.
[19] Tani E, Senba E, Kokumai S, Masuyawa K, Ishikawa T, Taohyamia M. Histamine application to the nasal mucosa induces release of CGRP and SP from peripheral terminals of trigeminal ganglion: a morphological study in the guinea pig. Neuroscience Letters 1990;112:1–6.
[20] Stephens LC, Ang KK, Schultheiss TE, King GK, Brock WA, Peters LJ. Target cell and mode of radiation injury in rhesus salivary glands. Radiotherapy and Oncology 1986;7:165–174.
[21] Stephens LC, Schultheiss TE, Price RE, Ang KK, Peters LJ. Radiation apoptosis of serous acinar cells of salivary glands and lacrimal glands. Cancer 1991;67:1539–1543.
[22] Stephens LC, Ang KK. Is substance P a mediator of radiation slaloadenitis?. International Journal of Radiation Oncology Biology Physics 1995;33(2):529–530.
[23] Church, MK, Benyon, RC., Rees, PH, et al. Functional heterogeneity of human mast cells. In: Galli, SJ, Austen, KF, editors. Mast Cell and Basophil Differentiation and Function in Health and Disease. Raven Press, New York, 1989, p. 161–170.
[24] Enerbäck L. Mast cells in rat gastrointestinal mucosa. I. Effects of fixation. Acta Pathologica et Microbiologica Scandinavica 1966;66:303–312.
[25] Donlon MA. Role of mast cell mediators in radiation injury and protection. Pharmacology and Therapeutics 1988;39:377–377.
[26] Castex N, Fioramonti J, Fargeas MJ, Bueno L. Role of 5-HT_3 receptor and afferent fibers in the effects of mast cell degranulation on colonic motility on rats. Gastroenterology 1994;107:976–984.
[27] Bjermer L, Hällgren E, Nilsson K, et al. Radiation-induced increase of hyaluronan and bibronectin in human bronchoalveolar lavage fluid from breast cancer patients is suppressed by smoking. European Respiratory Journal 1992;5:785–790.
[28] Fajardo LF, Steward R. Pathogenesis of irradiation induced myocardial fibrosis. Laboratory Investigation 1973;29:244–254.