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ORIGINAL ARTICLE

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Early immunohistochemical and functional markers indicating radiation damage of the parotid gland

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Abstract In order to evaluate the correlation between functional impairment and changes in the expression pattern of immunohistochemical antibodies in the early phase of radiation-induced dysfunction of salivary glands, eight rabbits were scintigraphically examined prior to and 24 h after irradiation with 15 Gy. The parotid glands were studied using HE-staining, Ki-67, α-smooth muscle actin (ASMA) and tenascin-C antibodies at every scintigraphic examination. The results demonstrated a significant alteration in the ^{99m}Tc-pertechnetate uptake in all irradiated glands. HE-staining showed no relevant impairment of salivary gland tissue in this early phase. Immunohistochemically, we observed a marked re-distribution of ASMA and tenascin-C as well as a reduction of the proliferating rate of acinar cells. This immunohistochemical change correlated with the functional impairment manifested scintigraphically. This study proves the possibility to assess disorders of salivary gland function with immunohistological antibodies as early as 24 h after irradiation and yields the prerequisites to prove the effects of radioprotective agents on salivary gland tissues.

Keywords Irradiation · Scintigraphy · Salivary glands · ASMA · Tenascin-C

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Introduction

Radiation-induced dysfunction of salivary glands still represents the main side effect of radiotherapy, influencing speech, chewing and swallowing in patients irradiated for head and neck malignancies. Many studies have tried to localise the target site of radiation in salivary gland tissue indicating cell membrane damage and interphase cell death, which have been reported to occur as early as 24 h following radiation and to stabilise after 72 h [23]. Mesenchymal alteration, including changes in the extracellular matrix such as laminin and collagen IV remodelling and fibrosis, may occur later [2, 8, 10].

Most studies using immunohistological methods to establish a sensitive quantitative parameter, which is able to predict morphological changes, have concentrated on the purely histological aspects, but have neglected simultaneous functional investigation. Therefore, the reliability of the parameters postulated in such studies has remained questionable. Apart from a single, conventional histological study on rhesus monkeys [23, 28], there is no evidence of early dysfunction-associated immunohistological changes of the salivary parenchyma. All other studies have investigated this aspect many weeks or months after irradiation [2, 10].

Therefore, this study investigated the correlation between early functional impairment and associated changes in the expression pattern of immunohistochemical antibodies of the different acinar cell lines in the parotid gland following irradiation. As an accurate and objective functional parameter of salivary secretion [4, 17, 18] we used salivary gland scintigraphy in an established rabbit experimental model combined with simultaneous conventional and immunohistological methods.

Material and methods

Animals and study design

Twelve healthy male and female New Zealand rabbits of 2.5–3.5 kg weight were used for the study. They were purchased from Charles-

River-Wiga, Sulzfeld, Germany and kept under laboratory conditions and alternating 12 h day/night rhythm; they received standard food (Altromin^{R}) and water ad libitum. All animals were acclimatised for at least 1 week before starting the study and then randomised into three groups. Experimental procedures were approved by local authority according to the current German law on the protection of animals.

Eight of the rabbits underwent a first scintigraphy as described below. One week later, four rabbits were irradiated with a single dose of 15 Gy and another four rabbits with 30 Gy, both under general anaesthesia (i.m. application) using a combination of 3 mg/kg (S)-Ketamin-hydrochloride (Ketanest-S^{R}) and 0.1 mg/kg Xylazin-hydrochloride (Rompun^{R}). Twenty-four hours after irradiation a second scintigraphy was performed with a subsequent excision of the parotid glands for histological examination. The remaining four animals underwent the same procedure without irradiation and provided control glandular tissue.

Quantitative salivary gland scintigraphy

Scintigraphy was performed in a prone position using a gamma camera (Picker 3000 XP) with LEUHR (low energy ultra high resolution) collimator. Dynamic studies were acquired with 90 frames and 30 s/frame) after intravenous application of 100 MBq $^{99m}TcO_4^-$ in 256×256 matrix (zoom 4). To induce flow of saliva, 0.01 mg/kg Carbachol (Doryl^{R}) was administered at 20 min. Percentage uptake of the administered activity was calculated within 10–19 and 36–45 min by summation of the appropriate frames and regions of interest of the parotid gland, and the difference of percentage uptake before and after administration of carbachol was calculated. Time-activity curves were registered and analysed.

The second scintigraphy was always performed 1 week after the first one in order to avoid any interaction or uptake disturbances by remains of the radiotracer injected previously.

The Salivary Ejection Fraction (SEF) was defined as follows: SEF = maximal uptake − rest activity/maximal uptake

Irradiation

Under general anaesthesia, x-ray irradiation was performed using 10 megavolt MEVATRON 74 Siemens teletherapy unit with a dose rate of 3 Gy/min. The rabbits were placed laterally and covered with a 1-cm thick tissue equivalent bolus material. An axial beam was directed from above towards the rabbit's head, extending from the retro-auricular region into the tip of the nose (field size 7.5×10 cm), thus including all salivary glands. Every side received 7.5 Gy and a whole exposure of 15 Gy was attained.

Surgical harvesting of salivary glands

Surgical exposure and excision of the parotid gland was carried out through a sagittal submandibular incision. The gland was divided into two portions; one was fixed immediately with 4% buffered formalin; another was shock frozen in liquid nitrogen and preserved for immunohistochemical examination.

Tissue preparation and immunohistochemistry

The biopsy samples were fixed in neutral phosphate buffered 4% formalin. After a minimum fixation of 48 h, the tissue was trimmed and processed by standard paraffin-embedding methods. Sections were cut at 4 µm, deparaffinised, and stained with hematoxylin and eosin (HE) to obtain conventional histological sections.

For immunohistochemical staining, monoclonal antibodies against the following proteins were utilised: alpha-smooth muscle actin (ASMA) (clone 1A4, diluted 1:40, Dako, Glostrup, Denmark); tenascin-C (TN2, diluted 1:25, Dako, Glostrup, Denmark) and Ki-67 (clone MM1, diluted 1:100, Novocastra, Newcastle, UK) [22]. The APAAP technique was used for the visualisation of the bound primary antibodies. The secondary rabbit anti-mouse antibody and the APAAP complex were diluted 1:50 (both from Dako, Glostrup, Denmark). Naphtol-AS-biphosphate (Sigma, St. Louis, MO, USA) and new fuchsin (Merck, Darmstadt, Germany) were used as substrate and developer, respectively. As negative control, the primary antibody was replaced by a non-immune serum. Considering the hot spots in the view field, Ki-67 antigen positive cells were evaluated per 300 epithelial cells in the studied glands. In order to evaluate the degree of ASMA loss per acinus, a semi-quantitative score was introduced as follows: No ASMA loss = 0; 25% loss of ASMA = +; significant ASMA loss >50% = ++; more than 75% ASMA loss = +++.

Ten view fields of each sample in every group were chosen accidentally and the degree of tenascin reaction in relation to the whole view field was evaluated using the professional Soft Imaging System software ANALYSIS^{R}. Values were expressed as mean ± SEM.

Data and statistical analysis

When proven to follow a normal distribution, values of tracer uptake and tenascin reaction were expressed as mean ± SEM and the two-sided *t*-test for paired and unpaired samples was used to compare scintigraphic and immunohistochemical data obtained prior to and 24 h after irradiation. For the evaluation of ASMA reaction, the non-parametric U-test was utilised. The SPSS software (Statistical Package for Social Sciences) was used for evaluation. An alpha level of $p<0.05$ was considered to be statistically significant.

Results

HE-staining

Apart from a mild secretory retention in the acinar cells of irradiated glands and an intracellular oedema, no specific changes could be observed. In irradiated glands the cellular borders were diminished and it was not possible to delimit adjacent cells; thus, the overall appearance resembled an undifferentiated homogenic cellular mass. Scattered vacuolopathy and a rise in the number of aberrant nuclei were noticed in irradiated acinar cells in both groups.

Ki-67 antigen

A significant change in the number of proliferating acinar cells of the parotid glands was assessed 24 h following 15 Gy. This ranged in the control glands from 1 to 3 nuclei in every "hot spot", and was significantly reduced in irradiated glands (0.1–0.8 nuclei; $p<0.01$).

Alpha smooth muscle actin (ASMA)

According to the known basket-like distribution of the myoepithelial cells in the acinar region and in the duct system [5], there were two different ASMA distribution patterns in the control parotid glands. While stained myoepithelial cells in the acinar region were noticed in a

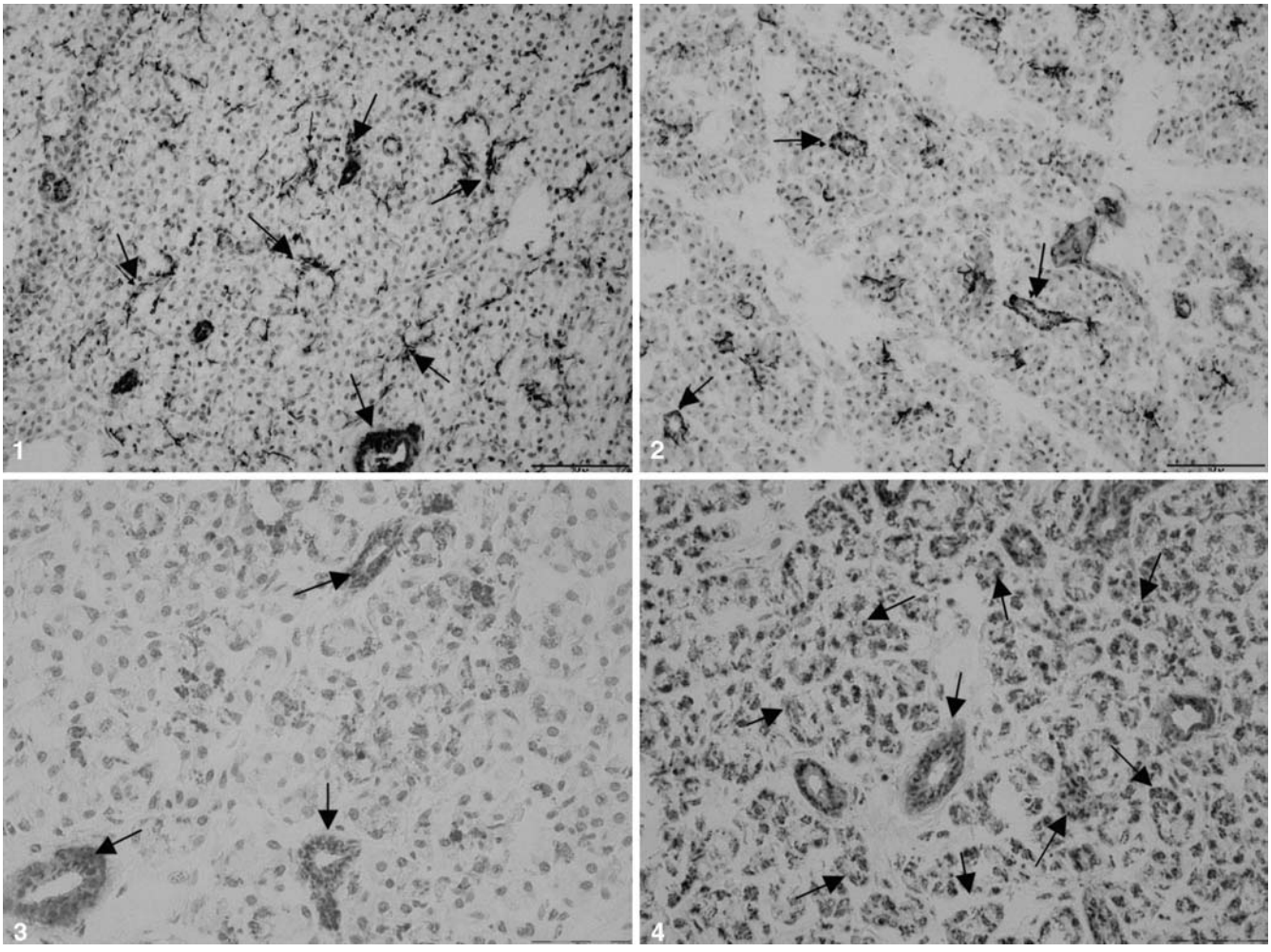

Fig. 1 Immunohistochemical expression pattern of ASMA in the control parotid gland. Notice the circular labelling of the intercalated ducts and the hem-like fashion in the acinar region

Fig. 2 Irradiated parotid glands show a marked loss of reaction in the acinar region, while the reaction of the myoepithelial cells remains unchanged around the intercalated ducts

Fig. 3 Tenascin-c expression is limited to the duct system and blood vessels as well in unirradiated glands; the acinar cells are not labelled

Fig. 4 Twenty-four hours following irradiation, the acinar cells display an excessive expression of tenascin-C

scattered hem-like pattern around the acinar cells and as a ring around intercalated ducts and blood vessels, myoepithelial cells of the intercalated and striated as well as around blood vessels were observed as a regular and continuous lining basally to the acinar cells and secretory ducts. In all regions, acinar cells themselves were not labelled (Fig. 1).

Compared with control glands there was a significant loss of ASMA immunostaining of the myoepithelial cells in irradiated glands. The myoepithelial cells were only evident as an irregular interrupted hem around some acini and were limited to only a few areas of acini and blood vessels in irradiated parotid tissue; however, ASMA reaction around the intercalated and striated ducts showed no significant alteration (Fig. 2). Original semi-quantitative data is shown in Table 1.

Table 1 Differences in the immunohistochemical distribution of ASMA and tenascin-C in control parotid glands and glands investigated 24 h following irradiation with 15 Gy

Antibody	Immunohistochemical remodelling		
	Control	After 15 Gy	*p*-value
ASMA	0	+ + (+)	<0.05
Tenascin-C	10.95±0.24%	87.33±1.06%	<0.01

Tenascin-C

In the control parotid glands, tenascin-C reaction was only limited to the basal membrane of the intercalated and secretory ducts and some acinar cells (Fig. 3). In irradiated parotid glands there was a re-distribution of tenascin-C staining. A partial loss of basal membrane staining was observed in addition to an excessive

Table 2 Alteration in the initial tracer uptake and Salivary Ejection Fraction (SEF) in the parotid glands 24 h following single dose irradiation with 15 Gy

	Tracer uptake			
	Initial uptake	After stimulus	SEF	*p*-value
Pre-radiation	0.293±0.011%	0.172±0.013%	41.29%	<0.01
After 15 Gy	0.215±0.014%	0.189±0.028%	12.09%	<0.05

deposition of tenascin-C in the acinar cells themselves (Fig. 4). This deposition could be seen in the cytoplysma of the acinar cells around the nuclei. The expression of tenascin-C in the myoepithelial cells could not be reliably evaluated, since no double staining had been performed.

The quantitative data of tenascin-C remodelling are shown in Table 1.

Scintigraphic data

Prior to irradiation, the initial ^{99m}Tc-pertechnetate uptake of the parotid glands ranged from 0.14 to 0.32%. After Carbachol application, a decrease of the rest activity was observed. In irradiated glands the remaining activity increased significantly (SEF=12.09%). Original data are given in Table 2.

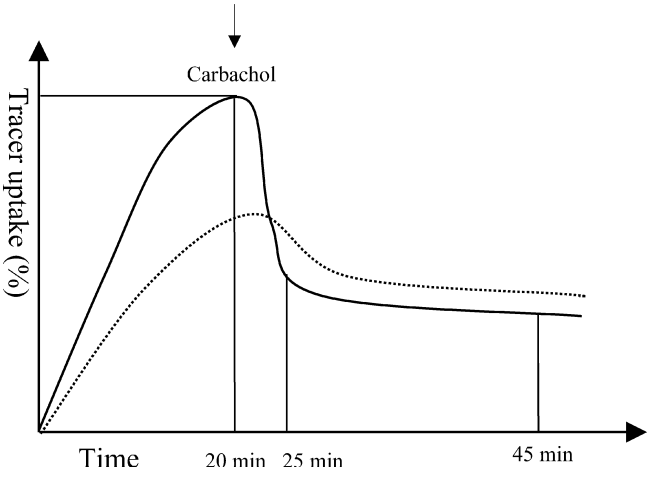

Fig. 5 Differences in the initial ^{99m}Tc-pertechnetate uptake and Salivary Ejection Fraction (SEF) of the control parotid glands (*solid line*) and the alteration 24 h following irradiation with 15 Gy (*dotted line*)

Discussion

Structural damage of salivary acinar cell was demonstrated in many studies as early as 1 h after radiation. These studies investigated either the morphological changes of the parenchymal cells and demonstrated only unspecific alteration which could also be observed in other diseases [7, 23] or accompanied changes in the composition of saliva [28]. They indicated changes in the cell membrane, aberrant deformation of nuclei, degranulation of the acinar cells or fibril-like condensation of mucous granules as consequences of radiation damage of salivary parenchyma.

The use of immunohistochemical methods in this field was limited to long-term investigations after irradiation [2, 10]. Although functional studies addressed this impairment early [19], neither scintigraphic nor immunohistological alteration in the early phase of radiation injury has—as far as we know—ever been investigated.

Simultaneous evaluation of both immunohistochemical and functional aspects gains a considerable value in this field since it illuminates the close relationship and may explain the pathogenesis. It also creates a sensitive parameter for radiation damage in salivary glands.

The use of salivary gland scintigraphy in evaluating impaired function of salivary glands after radiotherapy began in the early 1970s, but remained limited to the effects of radioiodine therapy in human and animal studies. Although the method has already been established, no uniform scintigraphic damage pattern has been introduced to explain the clinical observations. The findings range from obstruction [1] to a reduced uptake of the salivary parenchyma [4], to an altered stimulated salivary clearance [3], and to an altered salivary ejection fraction [27].

In this study we investigated the functional and the immunohistochemical alteration after irradiation of the parotid gland, since the serous type of salivary glands is more vulnerable to radiation injury and its function is severely affected [6]. Furthermore, it can be evaluated scintigraphically in this experimental model [11].

The scintigraphic results presented in our study reveal two crucial phenomena indicating radiation-induced dysfunction of the parotid gland (Fig. 5). The first one represents a significant reduction of the initial uptake of pertechnetate in the salivary parenchyma. This reflects a substantial disturbance in the capability of pertechnetate to enter the acinar cell. Since this process depends on the sharing mechanism of the $Na^+/K^+/Cl^-$ co-transporter [13], the basolateral membrane appears to represent one target in radiation damage. Recent studies have shown that the expression of tenascin, like other extracellular matrix molecules, may be suppressed by an intact basal membrane [15, 24]. This suggests that damage to the basal membrane of salivary acinar cells may in the same way implicate tenascin expression, which has been clearly demonstrated to be up-regulated in our study (Figs. 1, 2, 3 and 4). Similar radiation-induced remodelling in the expression of tenascin-C has recently been shown in the subepithelial layer of the conjuctival mucosa following brachiotherapy [12] as well as in skin samples following external beam radiation [20]. The study presented here is the first to investigate this phenomenon in an exocrine

parenchymal organ and the first to demonstrate associated functional impairment.

The other phenomenon represents the significant reduction of the SEF, implicated by the failure to release the radiolabelled saliva from the acini. In other words, the excretion process, which is normally initiated by the myoepithelial cells, is desintegrated [9]. This phenomenon could be attributed to a decrease or a non-response of the myoepithelial cells to the neurosecretory stimulus (Carbachol). This hypothesis is in accordance with the immunohistochemical findings highlighted in our study, since we observed a significant reduction of ASMA reaction in irradiated glands. ASMA is the main constituent of the thin filament in the myoepithelial cells which plays the main role in the contraction of the end piece epithelium and, consequently, in the release of salivary secretion. Therefore, failure of reactivity means an impairment of the contractile function of myoepithelial cells.

The redistribution of tenacin-C has been shown in various pathological processes, including vascular diseases [16] and salivary gland tumours [21, 22, 25], but also in the physiological involution of mammary glands [14, 26], indicating an association with cellular damage.

The detection of this redistribution in this study represents one of the earliest processes resulting from radiation injury in salivary glands. The subsequent ASMA loss is another sensitive marker for this damage. Both phenomena have taken place as early as 24 h after irradiation and are associated with pathognomonic functional changes.

Considering all aspects mentioned above, tenascin-C and ASMA re-modelling represent highly sensitive early markers of radiation-induced dysfunction of salivary glands. They may provide new functional surrogate parameters and prerequisites for further investigations in the field of radioprotection of salivary glands.

References

1. Albrecht HH, Creutzig H (1976) Salivary gland scintigraphy after radio-iodine therapy. Functional scintigraphy of the salivary gland after high dose radio-iodine therapy. ROFO Fortschr Geb Rontgenstr Nuklearmed 125:546–551
2. Bartel-Friedrich S, Friedrich RE, Lautenschlager C, Holzhausen HJ (2000) Dose-response relationships and the effect of age and latency period on the expression profile of laminin in irradiated rat mandibular glands. Anticancer Res 20:5221–5228
3. Blue PW, Jackson JH (1985) Stimulated salivary clearance of technetium-99m pertechnetate. J Nucl Med 26:308–311
4. Bohuslavizki KH, Klutmann S, Jenicke L, Brenner W, Feyerabend B, Henze E, Clausen M (1999) Radioprotection of salivary glands by S-2-(3-aminopropylamino)-ethylphosphorothioic (amifostine) obtained in a rabbit animal model. Int J Radiat Oncol Biol Phys 45:181–186
5. Dardick I, Rippstein P, Skimming L, Boivin M, Parks WR, Dairkee SH (1987) Immunohistochemistry and ultrastructure of myoepithelium and modified myoepithelium of the ducts of human major salivary glands: histogenetic implications for salivary gland tumors. Oral Surg Oral Med Oral Pathol 64:703–715
6. Dreizen S, Brown LR, Handler S, Levy BM (1976) Radiation-induced xerostomia in cancer patients. Effect on salivary and serum electrolytes. Cancer 38:273–278
7. El-Mofty SK, Kahn AJ (1981) Early membrane injury in lethally irradiated salivary gland cells. Int J Radiat Biol Relat Stud Phys Chem Med 39:55–62
8. Friedrich RE, Bartel-Friedrich S, Lautenschlager C, Holzhausen HJ, Moll R (2000) Dose response relations of collagen IV expression in irradiated submandibular gland of the rat. Mund Kiefer Gesichtschir 4:125–130
9. Garrett JR (1998) Myoepithelial activity in salivary glands. In: Garrett JR, Ekström J, Anderson LC (eds) Glandular mechanisms of salivary secretion. Karger, Basel, pp 132–152
10. Gustafsson H, Aalto Y, Franzen L, Thornell LE, Henriksson R (1998) Effects of fractionated irradiation on the cytoskeleton and basal lamina in parotid glands—an immunohistochemical study. Acta Oncol 37:33–40
11. Hakim SG, Lauer I, Kosmehl H, Sieg P (2002) The superficial mandibular gland of the rabbit: a new experimental model for scintigraphic evaluation of salivary glands. Int J Oral Maxillofac Surg 31:303–308
12. Heimann H, Coupland SE, Gochman R, Hellmich M, Foerster MH (2001) Alterations in expression of mucin, tenascin-c and syndecan-1 in the conjunctiva following retinal surgery and plaque radiotherapy. Graefes Arch Clin Exp Ophthalmol 239:488–495
13. Helman J, Turner RJ, Fox PC, Baum BJ (1987) 99mTc-pertechnetate uptake in parotid acinar cells by the Na+/K+/Cl- co-transport system. J Clin Invest 79:1310–1313
14. Jones PL, Jones FS (2000) Tenascin-C in development and disease: gene regulation and cell function. Matrix Biol 19:581–596
15. Jones PL, Boudreau N, Myers CA, Erickson HP, Bissell MJ (1995) Tenascin-C inhibits extracellular matrix-dependent gene expression in mammary epithelial cells. Localization of active regions using recombinant tenascin fragments. J Cell Sci 108:519–527
16. Jones PL, Crack J, Rabinovitch M (1997) Regulation of tenascin-C, a vascular smooth muscle cell survival factor that interacts with the alpha v beta 3 integrin to promote epidermal growth factor receptor phosphorylation and growth. J Cell Biol 139:279–293
17. Kohn WG, Ship JA, Atkinson JC, Patton LL, Fox PC (1992) Salivary gland 99mTc-scintigraphy: a grading scale and correlation with major salivary gland flow rates. J Oral Pathol Med 21:70–74
18. Liem IH, Olmos RA, Balm AJ, Keus RB, van Tinteren H, Takes RP, Muller SH, Bruce AM, Hoefnagel CA, Hilgers FJ (1996) Evidence for early and persistent impairment of salivary gland excretion after irradiation of head and neck tumours. Eur J Nucl Med 23:1485–1490
19. Mossman KL (1983) Quantitative radiation dose-response relationships for normal tissues in man. II. Response of the salivary glands during radiotherapy. Radiat Res 95:392–398
20. Riekki R, Jukkola A, Oikarinen A, Kallioinen M (2001) Radiation therapy induces tenascin expression and angiogenesis in human skin. Acta Derm Venereol 81:329–333
21. Shintani S, Alcalde RE, Matsumura T, Terakado N (1997) Extracellular matrices expression in invasion area of adenoid cystic carcinoma of salivary glands. Cancer Lett 116:9–14
22. Shrestha P, Sumitomo S, Ogata K, Yamada K, Takai Y, Yang L, Mori M (1994) Immunoreactive tenascin in tumours of salivary glands: evidence for enhanced expression in tumour stroma and production by tumour cells. Eur J Cancer B Oral Oncol 30B: 393–399
23. Stephens LC, Ang KK, Schultheiss TE, King GK, Brock WA, Peters LJ (1986) Target cell and mode of radiation injury in rhesus salivary glands. Radiother Oncol 7:165–174
24. Streuli CH, Bissell MJ (1990) Expression of extracellular matrix components is regulated by substratum. J Cell Biol 110:1405–1415

25. Sunardhi-Widyaputra S, van Damme B (1993) Immunohistochemical expression of tenascin in normal human salivary glands and in pleomorphic adenomas. Pathol Res Pract 189:138–143
26. Talhouk RS, Bissell MJ, Werb Z (1992) Coordinated expression of extracellular matrix-degrading proteinases and their inhibitors regulates mammary epithelial function during involution. J Cell Biol 118:1271–1282
27. van Acker F, Flamen P, Lambin P, Maes A, Kutcher GJ, Weltens C, Hermans R, Baetens J, Dupont P, Rijnders A, Maes A, van den Bogaert W, Mortelmans L (2001) The utility of SPECT in determining the relationship between radiation dose and salivary gland dysfunction after radiotherapy. Nucl Med Commun 22:225–231
28. Vissink A, Kalicharan D, S-Gravenmade EJ, Jongebloed WL, Ligeon EE, Nieuwenhuis P, Konings AW (1991) Acute irradiation effects on morphology and function of rat submandibular glands. J Oral Pathol Med 20:449–456