Effects of Fractionated Irradiation on the Cytoskeleton and Basal Lamina in Parotid Glands

An Immunohistochemical Study

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Acta Oncologica Vol. 37, No. 1, pp. 33–40, 1998

Cytoskeletal, cytocontractile and basement membrane proteins were studied using the immunofluorescence technique in the parotid gland in female rats after half-side fractionated megavoltage irradiation. The non-irradiated parallel-handled parotid glands served as controls. The qualitative expression of cytoskeletal proteins remained unchanged 10 days following irradiation compared to controls, i.e. cytokeratin was observed but not vimentin, desmin or GFAP (glial fibrillary acidic proteins). Six months after irradiation the cytokeratin expression adjacent to duct lumina was clearly stronger. Actin staining was more pronounced in the periphery of the acini. Ten days after irradiation no alterations of the basal lamina proteins, laminin and fibronectin, were detected. Six months post-irradiation laminin deposits were detected in areas where the entire acini had degenerated and had been replaced by fibrosis. An increased expression of fibronectin was also observed in the stroma at that time, reflecting an increased fibrosis. In areas where the acini remained, laminin immunofluorescence was mainly found in basal laminae of normal thickness, but the mean diameter of the acini seemed to have increased. This indicates a regeneration of acini and a restructuring of the basal lamina of the parenchyma.

Received 9 July 1997
Accepted 7 November 1997

Radiotherapy is known to cause a decrease in the production of saliva, with secondary dryness of mucous membrane. These effects have been attributed to a direct acinar loss (1). Although DNA in theory is the sensitive cornerstone and target of the effects of irradiation, other cell and tissue constituents such as cell membrane (2–4), vascular effects (5, 6), enzyme-containing secretory granules (7, 8) have been suggested as primary targets for the noxious radiation. It has also been shown that fractionated irradiation causes early effects on membrane-coupled secretory events, i.e. potassium release (3).

Morphological studies of salivary glands following irradiation have used mainly conventional light and electron microscopical techniques (6–10). None of the studies, as far as we know, have dealt with the influence on the cytoskeleton, i.e. microfilaments and intermediate filaments. The cytoskeletal proteins make up the framework and the stability of the cells. These proteins are important markers in tumour diagnostics (11–13).

The limited number of studies on the basal lamina have demonstrated a modest thickening of the basal membranes 10–15 days after a single irradiation dose of 800 rads (14), and convolution of basal membrane reflecting cellular death and acinar diminution have been seen (5). The basal lamina is an important structure, containing different structural proteins such as laminin, fibronectin, and collagen IV, separating epithelial tissue from the supporting stroma, from which the epithelial cells receive neuronal and hormonal stimuli as well as nutrition. Any structural alteration of the basal lamina would have an influence on these processes.

Therefore, we have used a system for unilateral fractionated megavoltage irradiation of the rat parotid gland for evaluating the morphological effects on several cytoskeletal and cytocontractile proteins and related the observed effects on basal membrane proteins to previous physiological studies.

MATERIAL AND METHODS

Animals

Twenty white albino female rats of Sprague Dawley strain (ALAB, Södertälje, Sweden) aged 8 weeks and weighing approximately 200 g were used. They were fed water and chow ad libitum and kept on a diurnal light programme.

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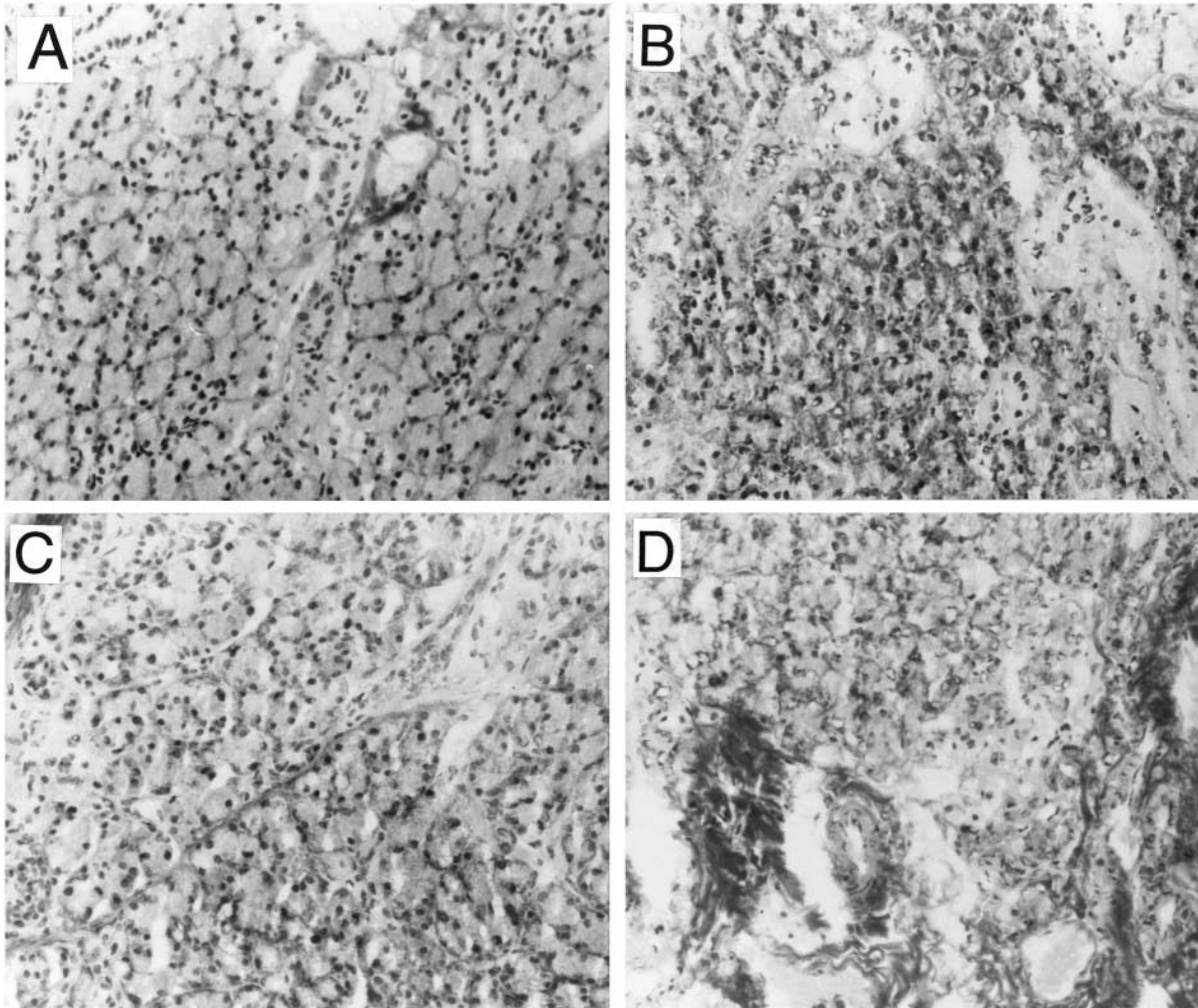

Fig. 1. Parotid gland tissue A. Control non-irradiated gland. B. Ten days after irradiation with 45 Gy. C. Six months after 30 Gy. D. Six months after 45 Gy. Ten days after irradiation no morphological alterations can be discerned (A, B). A progressive loss of acini can be seen 6 months after irradiation, indicated by areas of fibrosis (C, D). The remaining ducts are thinner than normal striated ducts (C). × 310 van Gieson stain.

Irradiation procedure

Irradiation of the rats was carried out on a medical linear accelerator—6MV. The rats were anaesthetized with Brietal 1–2 mg (Methohexital) and fixed in a plastic mould that held them firmly in position during the whole irradiation procedure. During this time the rats were observed through a TV camera. The total radiation field used on the accelerator was 8 × 20 cm when two rats were irradiated simultaneously. One side of the head—the reference side—was shielded with a thick lead block (80 mm). The geometrical margin between the radiation field and parotid gland was 10 mm in all cases and the distance between field edge and 95% dose level of this beam was 6–7 mm. Along with the maximum allowed position error, the parotid glands on the irradiated side achieved an insignificant dose. The absolute dosimetry was checked using an ionization chamber in a rat-like phantom and all scattering material in the field was kept constant. Doses of 6 or 9 Gy each were given on five consecutive days, Monday to Friday, up to a total dose of 30 or 45 Gy (10 rats in each group).

Tissue preparation

Ten days (10 rats) or 180 days (10 rats) after the end of the radiation period, the rats were anaesthetized with sodium pentobarbital and the irradiated and the contralateral parotid glands were rapidly excised and immediately frozen in liquid isopentane or prepared for electron microscopy.

Immunohistochemistry

Monoclonal antibodies against the following proteins were used (their sources are given in parentheses): GFAP (glial fibrillary acidic porteins) and neurofilament proteins

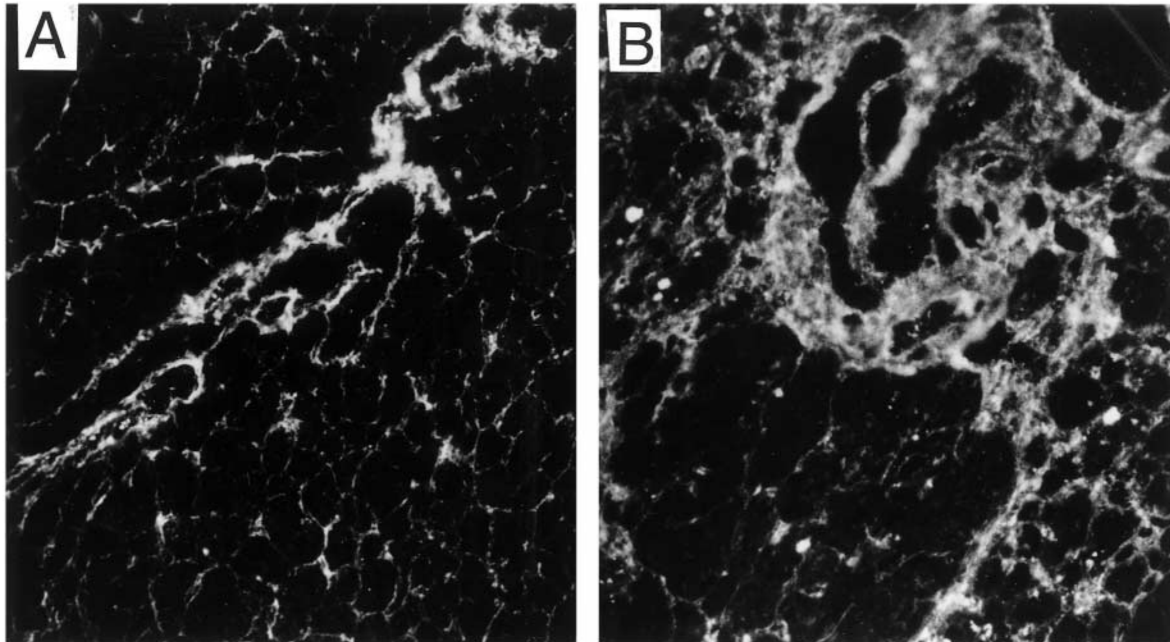

Fig. 2. Rat parotid stained with anti-fibronectin 180 days after irradiation with 45 Gy. A. Non irradiated control side. B. Irradiated side. A moderate increase in fibronectin indicating an increase in fibrosis can be discerned. × 320 Rhodamine stain.

(Dakopatts, Glostrup Denmark), Cytokeratin no. 7, (clone RPN 1162, Amersham Lab, Amersham, England), vimentin (clone Vim 9, Sanbio, Nistelrode, The Netherlands), desmin (EHS711) (15), foetal myosin (25EH2, Virtanen unpublished data) and plasmafibronectin (16).

A polyclonal antibody against laminin, a major component of the basement membrane, was obtained from E.Y. Labs (San Mateo, CA, USA) and F-actin was detected by rhodamine-phalloidine (Molecular Probes Inc., Junctions City, Oregon, USA).

Secondary antibodies were obtained from Dakopatts. Immunostaining was performed according to standard procedures as previously described (17).

Electron microscopy

Parotid gland specimens from irradiated glands and controls were fixed and embedded in parallel. Immediately after removal the parotid gland specimens were fixed in 3% glutaraldehyde in a 0.1 M phosphate buffer. After rinsing in buffer, the salivary gland specimens were post-fixed in 1% osmium tetroxide in the same buffer. After a cold buffer rinse the specimens were dehydrated in graded ethanol solutions and embedded in Epon 812. Semithin (1 μm) as well as thin (70 nm) sections were cut on an LKB Ultratome (Bromma, Sweden). Semithin sections stained with toluidine blue were used for light microscopical analysis. The thin sections were collected on naked copper grids and post-stained with uranyl acetate and lead citrate. A Jeol 1 200 EM electron microscope was employed for studying the fine structure of the glands of three rats 180 days after 45 Gy irradiation.

RESULTS

Overall morphology

In the non-irradiated side the well-known lobular structure with densely packed acini and ducts was seen in all specimens. No infiltration of fat cells or inflammatory cells occurred (Fig. 1A). On the irradiated side no changes were seen 10 days after 30 or 45 Gy irradiation (Fig. 1B), but 6 months after irradiation a loss of acini could be detected. Irradiation with 30 Gy resulted in a loss of approximately 15%, and the changes were unevenly distributed in the glands. The acini were replaced by minor duct-like structures and increased amounts of fibrous stroma (Fig. 1C). At 45 Gy a larger loss of most acini was observed. In some areas the remaining acini appeared to be normal, but at least 50% of epithelial gland tissue was made up of duct structures (Fig. 1D).

Immunohistochemistry

Fibronectin. Fibronectin was found as delicate strands between acini in the control sections (Fig. 2A). Ten days after irradiation no change in fibronectin fluorescence was seen. Six months after irradiation with 30 Gy a small increase in fluorescence between acini was seen. This was most pronounced in the vicinity of larger vessels and intercalated ducts, in accordance with the changes in overall morphology. After 45 Gy irradiation these changes were even more pronounced (Fig. 2B).

Laminin. In the non-irradiated parotid glands laminin staining was clearly visible in the acinar architecture in the gland (Fig. 3A). Equally-sized round acini were sur-

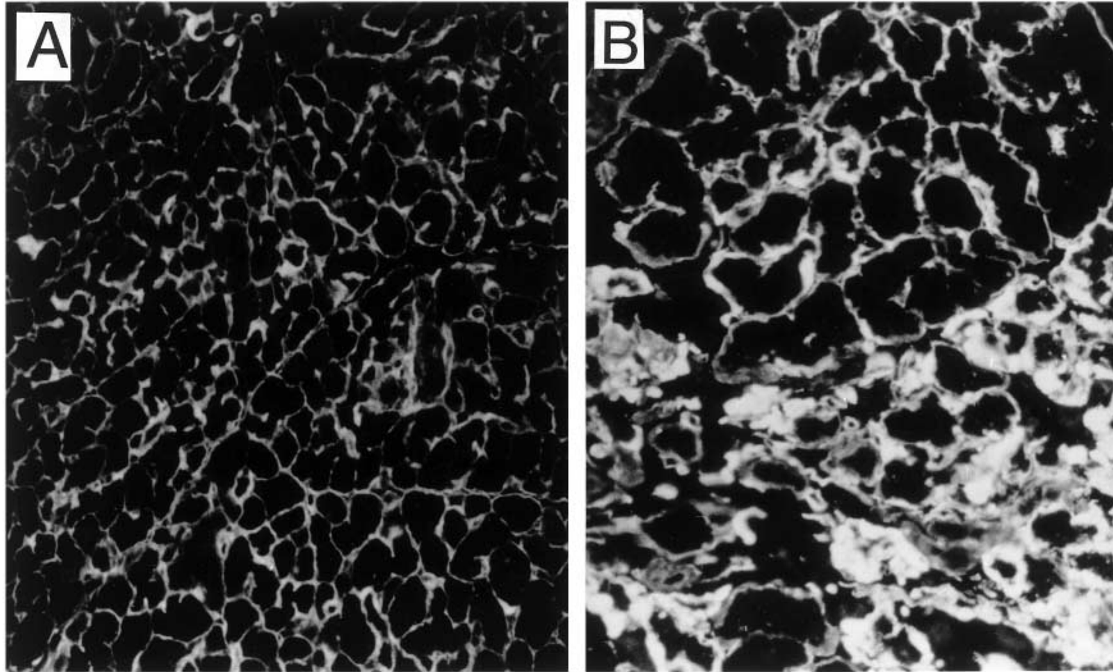

Fig. 3. Rat parotid stained with anti-laminin 180 days after irradiation with 45 Gy. A. Non irradiated control side. An insignificant increase in fibrous stroma can be seen. B. Large deposits of laminin can be seen, indicating loss of complete acini. The overall diameter of acini has decreased, and an increase in variation has occurred. × 310 Rhodamine stain.

rounded by a very distinct uniform fluorescent layer. The area surrounding the striated ducts and excretory ducts showed a thinner membrane of less staining and uniformity.

Ten days after irradiation no alterations were detected. On the other hand, 6 months later in the 30 Gy-irradiated rats, the parotid gland architecture was markedly altered. The remaining ducts and acini had a surrounding coating of laminin of the same shape as that on the control side. The loss of acini was clearly illustrated by large, dense deposits of fluorescent material. Furthermore, the overall size of acini had increased, and very clearly the size was no longer uniform, but differed markedly in certain areas. In the 45 Gy-irradiated glands, the few remaining ducts appeared normal in thickness and configuration of the surrounding laminin fluorescence in the basement membrane. In the adjacent fibrous stroma multiple strongly fluorescent deposits were seen, indicating the numerous lost acini. The few remaining acini displayed a variation in size, but were generally considerably enlarged compared with the opposite non-irradiated side (Fig. 3B).

Intermediate filament proteins. Vimentin, *desmin* and *GFAP* were at no time detected in any epithelial or myoepithelial cells, but desmin and GFAP were seen in some vessel and neural tissue, respectively. Vimentin was seen in fibroblasts which were present at a higher frequency in irradiated glands.

Cytokeratin immunoreactivity was particularly apparent to a considerable extent in the secretory luminal side of the epithelial cell of the non-irradiated tissue, at 10 and at 180 days (Fig. 4A). The same picture was also evident 10 days after 30 or 45 Gy irradiation.

An increased number of duct structures was observed 180 days following irradiation with 30 Gy, with cytokeratin 7 stain more evenly distributed along the whole cell membrane compared to the control side. The staining was most pronounced on the luminal side. These observations were still more pronounced after 45 Gy irradiation, with cuboid-like duct cells staining homogeneously (Fig. 4B).

Actin. Rhodamin-phalloidin staining, indicating the presence of actin in the non-irradiated glands, was most pronounced in the apical luminal side of acinar and duct cells, having its most prominent reaction in striated ducts, but also being observed in all other ducts and acini. The basal convoluted cell membrane of striated duct cells was moderately stained. The few myoepithelial cells were strongly stained. This pattern was virtually identical in the control and irradiated parotid glands 10 days after irradiation (Fig. 5A).

Most acini and ducts displayed a similar distribution of actin 180 days after irradiation with 35 Gy. However, in the areas in which a duct proliferation had appeared, more actin staining was seen just inside the basement membrane (Fig. 5B). This was still more pronounced after 45 Gy (Fig. 5C).

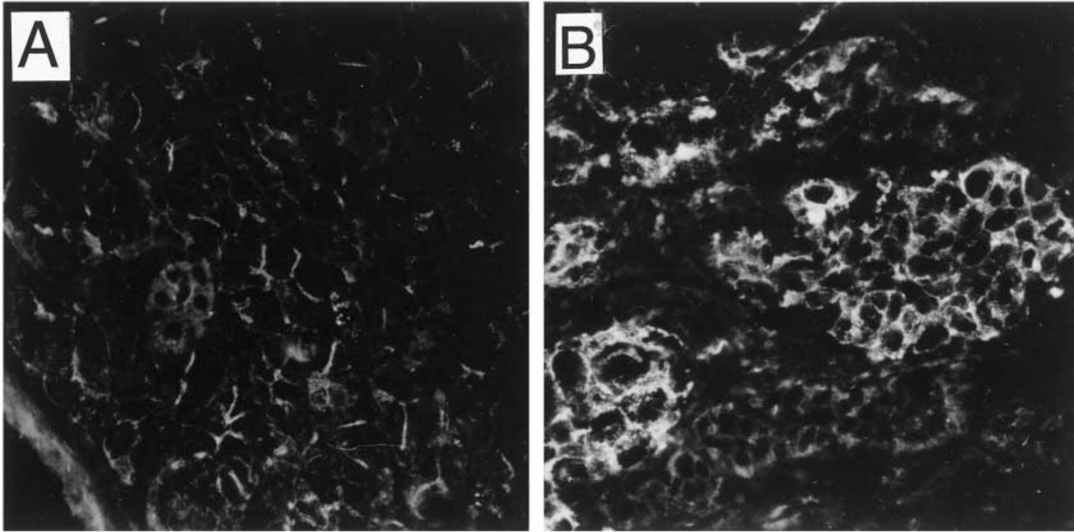

Fig. 4. Immunofluorescent staining of Ck 7 antibody 6 months after irradiation 5 × 9 Gy. A. Non-irradiated control side. Apical cell borders are sometimes strongly staining. B. Irradiated side. An increase in staining can be seen in certain areas where almost the whole cytoplasm is reactive. Rhodamine stain, × 200.

Ultrastructural morphology

Ten days after irradiation no alteration was found in gland morphology compared to the normal structure.

A loss of acini was noted 180 days after irradiation. The remaining acini appeared to be enlarged, sometimes with the cells lying in more than one layer in a disorganized manner. For the stroma and basilar lamina, no increase in basal lamina density, thickness or adjacent collagen structures could be seen in duct cells, acinar cells or myoepithelial cells. No increase in the number of myoepithelial cells was seen either. The basal lamina surrounding the vessels seemed to be unaffected. In the stroma, abundant debris and cellular remnants could be seen, particularly after 45 Gy, corresponding to the fluorescent deposits in the immunostained sections.

DISCUSSION

The present study on long-term effects (180 days after irradiation) did not demonstrate the direct effects on basement membrane but merely indirect effects of irradiation. The thickness did not change and no disruption in continuity or duplication was seen. The laminin stain was stronger around the acini than around the striated ducts, which indicates a loss of acini, where some acini have vanished completely, leaving only the vestiges of basement membrane as a densely immunostainable deposit, while others are diminished in diameter or transformed into ducts. The findings of these huge acini may indicate a regeneration, as has recently been suggested (18). In the acute phase (10 days) of the study no influence of irradiation was seen at the light microscopical level, which implies that the protein structures are relatively resistant to irradiation, and the turnover of proteins is slow or the synthesis or maintaining of these protein structures is not affected. This is in contrast to the earlier observations of altered expression of other substances, such as neuropeptides, which displayed an increased expression days after irradiation (19, 20).

Previous morphological studies of irradiated salivary glands have not included the cytoskeletal or cytocontractile proteins. In the present study cytokeratin 7 was increased in the duct cells after 6 months in proportion to the radiation dose and the pattern of staining was more diffuse compared to controls. In epithelial cells in general the intermediate filaments are composed of only cytokeratins (17, 21). However, GFAP have been found in a small percentage of myoepithelial cells in salivary glands (22, 23). In foetal parotid (17), vimentin can be found in certain myoepithelial cells but the findings in adult myoepithelial cells have been divergent (24, 25). Furthermore, besides cytokeratins, parotid tumour cells can contain vimentin, desmin and glial fibrillary acidic proteins (22, 23, 25). The cytoskeleton of all cells is very stable against alterations in the environment; even after neoplastic transformation, the protein composition of the different filaments often remains intact. This is of great practical importance in tumour pathology. (11, 12, 26–28). No previous systematic study on the radiosensitivity of these structures has to our knowledge been carried out. In the salivary gland tumours, however, no alterations were found in the intermediate filament protein pattern after preoperative radiotherapy (24).

The cytokeratin 7 expression in the untreated glands is positioned in the highly specialized secretory surface of the gland. A dedifferention to a more immature cell type would probably mean a loss of expression of such special-

ized proteins. The persistence of this expression after irradiation thus indicates remaining cytoskeletal structures which may be involved in the secretory processes. Evidence has been seen of an intact cellular response with amylase exocytosis on adrenergic stimuli 6 months after irradiation of up to 45 Gy (3, 4). An increase in cytokeratin 7 and a lack of directional specialization after irradiation are puzzling from the point of differentiation. In the regenerating acini a certain loss of organization evidently occurs, as acinar cells must lie in several layers between the main acinar lumen and the basement membrane, if no branching of the lumina occurs, due to the increased size of the acini.

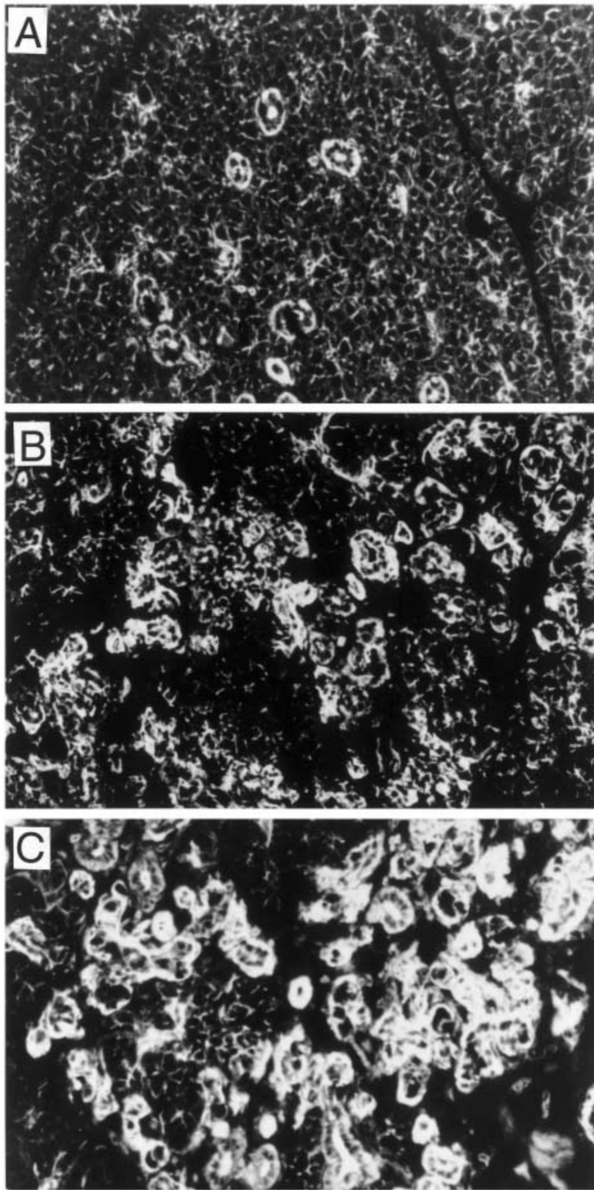

Fig. 5. Immunofluorescent staining 6 months after irradiation. A. Control gland (5×6 Gy). Rhodamine-phalloidin immunoreactivity can be seen in the apical cell borders both in the acini, ducts and in the myoepithelial cells. B. Gland irradiated 5×6 Gy. Apical cell borders are still staining but the peripheral parts of the duct are increased in reactivity. C. Gland irradiated 5×9 Gy. The increase in stainability is sometimes enormous, blurring the pictures. $\times 140$.

In contrast to previous studies on rat parotid glands, an abundance of basal lamina was seen 60 days after irradiation with 3.2 kR with and without radio protection by WR-2721 (5). It was found that in areas where larger numbers of cells were lost, the basal lamina formed highly convoluted and collapsed structures, which were often apparently empty and additional layers were found surrounding some capillaries. The absence of such findings in the present study may indicate the timing of the post-radiation processes; After 60 days the progressive death of acinar cells continues, whereas after 180 days cell death rates are back to normal. Regeneration of acini and ductal structures requires the reorganization of basement membrane into a normal state. In the 45 Gy-radiated-rats an enlargement of acinar size was seen, which indicates a regeneration of acinar structures with a focally abnormal acinar cell renewal. This further strengthens the theory of a regeneration and remodulation of an indirectly affected basal membrane within the vital parts of the gland tissue.

An effect of irradiation on the vessel structures would correspondingly affect transport between the vascular and intercellular spaces. The basement membrane divides the epithelial part of the gland from its stroma. The autonomic innervation of the rat parotid has its terminal axon varicosity interposed between acinar cells and basal lamina as well as interstitially (28). A thickening of the basement membrane would have an influence on the effects from the latter type of innervation. Likewise, a hormonal effect from vessels as well as all kinds of nutrition would be affected.

The increase in fibrinogen after high doses of irradiation in all likelihood displays the fibrosis that accompanies the loss of acini. Presumably this fibrosis affects transportation of hormones, gases and nutrients to and from the acinar cells but does not affect the neural mechanisms adjacent to the acinar cells, as many axon terminals lie on the epithelial side of the basal lamina (28).

A persistent actin staining in the luminal part of the acinar and duct cells was seen, but also an increase of actin staining in the basal part of the duct cells, especially striated ducts. The presence of an apical layer of actin in secretory epithelia is well known and has also previously been described in salivary glands (29). No definite function has been proven but some kind of participation in the secretory process seems plausible. An increase in actin content has been found in salivary gland cells in various types of pathology. In Sjögren's syndrome the myoepithelial cell islands are found to contain more actin-containing cells (30). Furthermore, many tumour cells contain more actin than the cell of origin (11). The increased amount of actin has also been attributed to the origin of the tumour cells in myoepithelial cell or precursor. None of the ultra-

structural studies on irradiated salivary glands have described any alterations of the myoepithelial cells (18). Our findings of an increase in basally positioned actin in intercalated ducts thus cannot be explained as a simple hyperplasia or hypertrophy of myoepithelial cells, but must have occurred in the duct cells and in the acinar cells.

To summarize, one of the objects of this study was to investigate the short and long-term effects of irradiation on the apical cell luminal surface of the parotid acinar cells in the rat. Neither the actin content nor the expression of cytokeratin 7 seemed to be affected, indicating a possibility of remaining secretory activity, which has been shown in secretory studies following adrenoceptor stimulation (4). In contrast, the amount of both these proteins increased in the lower part of these cells and also in duct cells. No increase in myoepithelial cells was found. Furthermore, changes of the basal membrane were seen, reflecting a restructuring of the parenchymal gland tissue but no alteration indicating an increase in barrier function in either the vascular or epithelial side. The alterations in basal membrane structure thus do not seem to be a factor of importance for the change in salivary gland secretory response long after irradiation.

ACKNOWLEDGEMENTS

We express our gratitude to Ann-Louise Grehn for skilful technical assistance. This study was supported by grants from the Swedish Medical Research Council (12X-3934), the Lions Fund, and the Medical Faculty of the University of Umeå.

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