Abstract: The present study was done to develop a useful experimental model for analysis of the effects of physiologically active substances on atrophy and regeneration of salivary gland acinar cells. Resection wounds (diameter, 3 mm) were made in the submandibular glands of 8-week-old Wistar rats (n = 24) for histochemical examination on Days 3, 5, 7, 10, 14, and 21 after implantation of a gelatin-based hydrogel sheet. The results showed that the sheet had nearly disappeared by Day 10. Regions around the resection wounds were classified as normal, atrophic, or necrotic. In atrophic regions, acinar cells atrophied after resection, and few acinar cells were observed on Day 7. On Days 5-7, striated and granular ducts resembling duct-like structures. On Day 10, newly formed acinar cells were confirmed by increased periodic acid-Schiff staining, and a greater number of mature cells was present thereafter. In necrotic regions, acinar and ductal cells were destroyed, and scattered enucleated acinar cells and duct-like structures were present, on Day 3; newly formed acinar cells were observed on Day 10. Thus, the experimental model demonstrated atrophy and regeneration of the submandibular gland and enabled analysis of the effects of sustained release of physiologically active substances contained within an implanted sheet.

Keywords: atrophy, necrosis, regeneration, resection, submandibular gland

Introduction

Salivary gland tissue can be damaged by conditions such as sialadenitis, sialolithiasis, trauma, and radiation therapy, which can lead to degeneration, atrophy, and necrosis. Methods to examine these pathological conditions include surgical resection [1-3], duct ligation [4-6], radiation [7,8], laser irradiation [9,10], and histopathological and immunohistochemical analysis.

Milstein [11] and Hanks et al. [1] reported that, after partial resection of salivary glands, newly formed acinar cells are generated from resection margin ducts during regeneration. However, Boshell et al. [2] and Batsakis et al. [12] found that epithelial cells may proliferate at intercalated ducts. A duct ligation model revealed that acinar cells regenerate through pathways other than proliferation of residual acinar cells, suggesting that the epithelia of intercalated ducts and duct-like structures contain cells that can regenerate into acinar cells [13]. Furthermore, regeneration of acinar cells around a laser irradiation-induced necrotic wound was initiated in epithelial cell nests of duct-like structures [9,10]. However, the mechanisms by which progenitor cells proliferate and differentiate into acinar cells remain unclear.

Immunohistochemical studies of atrophy and regeneration of salivary glands in the above experimental systems revealed localization of growth factors such as epidermal growth factor [14,15], fibroblast growth factors (FGFs) [16], and nerve growth factor [14]. However, their roles remain unclear, and the long-term effects of procedures such as intra-ductal retrograde injection of growth factors are unknown [17]. Tabata et al. [18] recently examined whether a gelatin-based hydrogel sheet could enable sustained release of physiologically active substances and found that the sheet allowed (1) topical administration of physiologically active substances, (2) stabilization of physiologically active substances that were easily degraded and inactivated in vivo, and (3) sustained release of physiologically active substances for approximately 2 weeks.

Therefore, in the present study, a circular resection wound was prepared by a biopsy punch of a rat submandibular gland, into which a sustained-release sheet was implanted. The morphology of healing glandular tissue was examined to determine the usefulness of this experimental model for analyzing the effects of physiologically active substances on atrophy and regeneration of acinar cells.

Materials and Methods

Animals

Twenty-four 8-week-old male Wistar rats (Sankyo Labo Service Corpora-
tion, Tokyo, Japan) were used in this study. The rats underwent 1-week adaptation to the environment at the Animal Testing Facility of Nihon University School of Dentistry. All procedures were conducted in accordance with the Animal Experiment Guidelines of the Nihon University School of Dentistry (approval number: AP16D045). During the study, no rats died of infection.

Model of submandibular gland resection

For general anesthesia, a mixture of three anesthetics—medetomidine hydrochloride (0.15 mg/kg Domitor, Nippon Zenyaku Kogyo Co., Ltd., Koriyama, Japan), midazolam (2 mg/kg Sand, Sandoz K.K., Kamiyama, Japan), and butorphanol tartrate (2.5 mg/kg Butorphanol, Meiji Seika Pharma Co., Ltd., Tokyo, Japan)—was intraperitoneally administered to each rat, in combination with isoflurane (2% Forane inhalation anesthetic, AbbVie GK, Tokyo, Japan). Before surgery, lidocaine hydrochloride (2-4 mg/kg 1% lidocaine injection, Nissin Pharmaceutical Co., Ltd., Tendou, Japan) was applied for local anesthesia in the submandibular region. A 2-cm skin incision was made in the middle of the anterior neck to expose the submandibular gland, followed by pull-through resection of the caudal region in the submandibular gland with 3-mm disposable biopsy punches (Biopsy Trepan, Kai Industries Ltd., Seki, Japan) (Fig. 1A). A gelatin-based hydrogel sheet (MedGel, MedGEL Corporation, Tokyo, Japan) of the same size was implanted into the resected area (Fig. 1B). MedGel contains cross-linked, water-solubilized gelatin (a gelatin-based hydrogel sheet) that allows sustained release of a physiologically active substance over 2 weeks.

Histochemical observation

Rats were euthanized with carbon dioxide on Days 3, 5, 7, 10, 14, and 21 after surgery, and their submandibular glands were excised. The glands were immersed fixed in 4% paraformaldehyde for 24 h, then embedded in paraffin blocks, as described previously [19]. The tissues were sliced at a thickness of 8 μm with a microtome and then stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS) stain.

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Results

HE staining images of submandibular glands, removed at 3-21 days after resection, are shown in Fig. 2Aa-f. The implanted sheet gradually shrank, beginning on Day 3 (Fig. 2Aa, b), and was approximately one-third its original size on Day 7. The mesh structure of the sheet also became thinner, and fibroblast invasion was observed (Fig. 2Ac). By Day 10, the sheet had mostly disappeared but was partially recognizable (Fig. 2Ad). However, beginning on Day 14, the sheet could not be recognized in any of the rats (Fig. 2Ae, f). Morphologically, areas around the MedGel sustained-release sheet were classified as follows: a cranial region unaffected by resection; a cranial region adjacent to the sheet, which exhibited atrophy and regeneration; and a caudal region adjacent to the sheet, which exhibited necrosis. The positional relationships between these regions and the resection site are depicted in Fig. 2B. HE and PAS staining images of atrophic and necrotic regions on Days 3-21 after surgery are shown in Fig. 3a-f and Fig. 3g-r, respectively.

Atrophic region

On Day 3 (Fig. 3a, g), slightly atrophic acinar cells were noted, with small vacuoles scattered in the cytoplasm and a slight reduction in the number of secretory granules. In addition, some PAS-stained atrophic acinar cells were present. Growth of granulation tissue was observed surrounding the duct, along with slightly obscure basal striations. On Day 5 (Fig. 3b, h), terminal acinar cells exhibited further cytoplasm atrophy and deformation; the numbers of PAS-positive cells were also reduced, as compared with those on Day 3. Additional growth of granulation tissue was observed surrounding the duct-like structures, which could not be distinguished from striated and granular ducts.

On Day 7 (Fig. 3c, i), the terminal acinar cells were mostly atrophied and had disappeared. PAS staining of acinar cells was weaker than on Day 5. In the central part of the epithelial nest, squamoid cells with relatively bright cytoplasm were observed. In the ducts, nuclei exhibited weak hematoxylin staining, with cytoplasm atrophy and deformation, indicating a duct-like structure. On Day 10 (Fig. 3d, j), newly formed, undifferentiated, PAS-positive acinar cells were observed at the tip of the duct-like structure. In addition, duct-like structures with a clear lumen were scattered adjacent to undifferentiated acinar cells. On Day 14 (Fig. 3e, k), immature acinar cells were observed alongside mature cells at the tips of intercalated ducts, which were thinner than the duct-like structures observed on Day 10. On Day 21 (Fig. 3f, l), acinar cells were mostly mature with PAS-positive granules, and intercalated ducts with a clear lumen had gradually aligned, forming a relatively distinct leaflet.

Necrotic region

On Day 3, a necrotic region was observed at the caudal side of each resection site. The resection site was slightly edematous, which obscured the boundary between the necrotic region and its surrounding region (Fig. 2Aa). Acinar and ductal cells were mostly destroyed, and the morphol-
ogy was obscured by enucleated acinar cells and scattered degenerated ducts (Fig. 3m). On Day 5, the necrotic region was slightly smaller than on Day 3 (Fig. 2Ab). The acini had further atrophied, as compared with their morphology on Day 3, while fibrous connective tissue increased. In addition, epithelial cell nests consisting of squamoid cells appeared with a few duct-like structures that exhibited granules with weak PAS-positive staining (Fig. 3n).

On Day 7, the necrotic region had shrunk (Fig. 2Ac). As compared with granulation tissue on Day 5, more epithelial cell nests were present on Day 7. Squamoid cells with relatively bright cytoplasm were present in the central portions of epithelial cell nests. The nests varied in size and shape (i.e. round to dendritic) and were followed by a duct-like structure with a continuous lumen (Fig. 3o). On Day 10, the necrotic region further decreased in size (Fig. 2Ad). The necrotic region contained a large amount of fibrous connective tissue, which had a duct-like structure comprising cubic epithelial cells. In addition, immature acinar cells with PAS-positive granules were located adjacent to the duct-like structure (Fig. 3p). On Days 14 and 21, the necrotic region further decreased in size and exhibited fibrotic granulation tissue (Fig. 2Ae, f). Many duct-like structures were found in the necrotic region. As compared with the number of acinar cells on Day 10, greater numbers of acinar cells with PAS-positive granules adjacent to the duct-like structures were present (Fig. 3q, r).

Fig. 3  HE (a–l) and PAS staining (g–r) images of atrophic (a–l) and necrotic (m–r) areas on Days 3 (a, g, m), 5 (b, h, n), 7 (c, i, o), 10 (d, j, p), 14 (e, k, q), and 21 (f, l, r). On Day 5, duct-like structures appeared in atrophic areas (blue arrows: b) and necrotic areas (blue arrowheads: n). On Day 7, epithelial nests were observed in atrophic areas (green arrows: c) and necrotic areas (green arrowheads: o). On Day 10, newly formed, undifferentiated, PAS-positive acinar cells (yellow arrows: j and p) were observed at the tips of duct-like structures. Scale bar = 100 μm.
Discussion

The objective of the present study was to develop an experimental model to examine the effects of physiologically active substances that affect atrophy and regeneration of acinar cells after salivary gland resection. Submandibular gland tissue was resected with a 3-mm biopsy punch, and a sustained-release sheet containing the physiologically active substance was implanted to observe morphological changes in gland tissue on Days 3, 5, 7, 10, 14, and 21.

On Day 7, the sustained-release sheet implanted in the resected region was approximately one-third its original size, and the mesh structure of the sheet was thinner. By Day 10, the sheet had mostly disappeared but remained recognizable. However, on Day 14, the sheet was not recognizable in any rat. The manufacturer explained that if the sheet were immersed in a physiologically active substance, it would gradually release that substance for 14 days. However, in the present experimental model, the sheet was recognizable until Day 10. Therefore, growth factors were immersed in the sheet, resulting in sustained release for up to 10 days.

In submandibular gland tissue, three morphological regions (normal, atrophic, and necrotic) were observed near the resected region. In the atrophic region, acinar cells were atrophied and fewer in number after resection, and the number of acinar cells was lowest on Day 7. On Days 5-7, duct-like structures had replaced striated and granular ducts. Beginning on Day 10, terminal acinar cells gradually regenerated and PAS staining increased. In addition to mature acinar cells, immature cells were observed at the tip of the duct-like structures. On Day 21, atrophic regions had an appearance similar to that of normal glandular tissue. Milstein [11] reported thickening of the lining of the main duct and branching of granulation tissue on Day 5 after partial resection of the submandibular gland; this was followed by division of the duct branches and differentiation into acinar cells on Day 7. Hanks et al. [1] reported degeneration and necrosis in the central lobe of the submandibular gland, as well as marked growth of epithelial cells from undeveloped ductal cells on Days 7-14 after resection. These findings are consistent with those of the present study, despite the use of different resection methods, which suggests that acinar cells can regenerate from remaining ducts.

In this study, a necrotic region due to vascular insufficiency after resection was observed in the caudal region adjacent to the sheet. On Day 3, the acinar and duct cells were mostly destroyed, and the morphology was obscured by enucleated acini and scattered duct-like structures. On Day 10, newly formed acinar cells appeared, and their number gradually increased until Day 21. Takahashi et al. [9] reported regeneration of the necrotic region after YAG laser irradiation and growth from residual duct-like structures around the necrotic nest on Days 1-3 after irradiation, which rapidly became highly proliferative conduits. Beginning on Day 7, undifferentiated polygonal cells were differentiated into immature acinar cells. On Day 28, small acinar cells were mixed with mature cells. As compared with the results of Takahashi et al. [10], the present findings showed that newly formed acinar cells appeared and matured more slowly in the necrotic region, suggesting excessive damage to the necrotic region during resection. However, the overall results were similar, which indicates that longer observation of regeneration of acinar cells in the necrotic region is required.

Takahashi et al. [9] reported the presence of many epithelial cell nests consisting of squamous cells in granulation tissue, which formed around the necrotic region after laser irradiation. They presumed that the nests might differentiate into duct-like structures and that striated and intercalated ducts and acini could regenerate from these duct-like structures. Hanks et al. [1] described squamous metaplasia, which may be a tissue similar to that of squamous cell nests. In the present study, epithelial cell nests were observed at the boundaries between atrophic and necrotic regions; these nests were adjacent to a duct-like structure with a clear lumen, as reported by Takahashi et al. [9, 10], suggesting that acinar cells in the necrotic region could regenerate from the duct-like structures.

In this study, a circular resection wound was created with a biopsy punch, and a sheet of the same size was implanted into the wound. The resection site shrank over time, and the mesh structure of the MedGel sheet had begun collapsing by Day 7, in conjunction with invasion of surrounding fibrous connective tissue. The resection wound was visible on Day 10 but began to be difficult to discern on Day 14. These findings suggest that the resection wound was replaced by fibroconnective tissue, without regeneration of salivary gland tissue, and are generally consistent with those of previous reports [2,11]. FGF-2/FGF receptor-1 signaling was found to promote atrophic rat submandibular gland repair [17]. There is a report of localization of FGFs and FGF receptors during atrophic rat submandibular gland regeneration [16], and it has been suggested that epidermal growth factor affects atrophy regeneration [14,15]. However, the process of identifying the function of these growth factors is one of atrophy/regeneration, and no study has evaluated the effect on the necrotic region. The present findings indicate that the role of physiologically active substances in the necrotic region can be studied with MedGel, because regeneration of the acinus in the necrotic region, as well as in the atrophic region, was confirmed. Thus, the present experimental model suggests that implantation of a sustained-release sheet containing a physiologically active substance at a resection site appears useful for analysis of the effects of that substance.

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Conflict of interest

The authors declare no conflict of interest.

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