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Microcalcifications in stone-obstructed human submandibular gland are associated with apoptosis and cell proliferation

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Abstract

Objective—Human submandibular gland (SMG) stones are associated with inflammation, fibrosis and microcalcifications in the surrounding tissues. However, there is little information about the accompanying cell injury-repair process, apoptosis, and cell proliferation. The purpose of this study was to investigate such an association and its clinical significance.

Design of Study—Mid-gland paraffin sections of human SMGs (“stone glands”) and normal SMGs (“non-stone glands”) were subjected to stains for general histology (hematoxylin and eosin), fibrosis (Masson’s trichrome), and calcification (alizarin red) and to immunohistochemistry for proliferative activity (Ki-67), and apoptosis (Caspase-3). Tissues were assessed for areas of inflammation, calcium deposition, and fibrosis, and for cycling and apoptotic cells.

Results—Acini were atrophic and proportionately fewer in lobules with fibrosis in stone glands. Additionally, stone glands had intraluminal calcifications (microliths) in scattered excretory and striated ducts and blood vessel walls. Areas of inflammation and fibrosis were small and uncommon, and calcifications were not seen in non-stone glands. Proliferating and apoptotic cells

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Competing Interest: There is no conflict of interest related to the submitted manuscript.

Ethical approval

Specimens are formalin fixed paraffin embedded (FFPE) de-identified tissue section (biopsy sample, not individually identifiable to any person) from Georgetown University tissue bank through an exempt Institutional Review Board (IRB) protocol (protocol number: 2010-423; IP: Bandyopadhyay). This IRB exemption was also approved by the IRB and the Research and Development committee of the Washington DC VA Medical Center.

were common in the main duct of stone glands where ciliated and mucous cell hyperplasia and stratified squamous metaplasia had occurred, uncommon in the main duct of non-stone glands, and uncommon in all other parenchymal elements of both stone and non-stone glands.

Conclusion—Stone obstruction in the main excretory ducts of SMG resulted in progressive depletion of acini from proximal to distal lobules via calcification, inflammation, fibrosis, and parenchymal cell atrophy, apoptosis and proliferation. Interlobular duct microliths contributed to this depletion by further provoking intralobular inflammation, fibrosis, and acinar atrophy.

Graphical abstract

Keywords

Calcification; fibrosis; cell proliferation; apoptosis; salivary gland stone

1.0 INTRODUCTION

Calcium phosphate (CaP) crystals incite the process of calcium stone formation as well as ectopic calcification. Such calcification displays some similarities with the mechanisms of bone formation, where bone related factors have been shown to play an important role in vascular and renal calcification (Schweighofer et al., 2016; Jia et al., 2014). Moreover, calcification can be the body's protective response to injury, as well as part of a natural inflammatory reaction to infection, trauma, or autoimmune disorders. Tumor tissues (either cancerous or noncancerous) also have been shown to contain areas of calcification (Khan et al., 2010), however the link between those phenomena to support a mechanism is unknown. Similarly, the inflammatory response can be triggered by ectopic calcifications (Fukuyo et al., 2014), which can then release several inflammatory cytokines such as IL-6 and TNF-alpha to induce the signaling pathways leading to osteoblast differentiation, essential for calcium biomineralization (Fukuyo et al., 2014; Watson et al., 1994). Additionally, calcium crystals are able to generate a proinflammatory response (Watson et al., 1994; Harrison, Triantafyllou, Baldwin, & Schäfer, 1993) in macrophages, which ingest CaP crystals, secrete the cytokines TNF-alpha, IL-1-beta, and IL-8 (Nadra et al., 2005). Since CaP crystals are

able to trigger the secretion of inflammatory cytokines which themselves are able to trigger the formation of calcifications, it is likely that there is a positive feedback loop between calcification and inflammation (New & Aikawa, 2011).

Likewise, links between calcification and other physiological conditions such as fibrosis have also been described, particularly in renal tissue (Evan et al., 2005), where CaP stones have been linked to loss of nephrons (Coe, Worcester, & Evan, 2016). Moreover, calcium crystals are also associated with fibrosis, calcification and cell proliferation (Weon et al., 2012; Hayes, Brodie, O'Doherty, & Quinn, 2013; Morgan, Cook, & McCarthy, 2005). Furthermore, increased cell turnover (i.e., increased proliferation and apoptosis) can be a contributing factor in tumorigenesis (Liu, Edgerton, Moore, & Thor, 2001). However, such cell injury-repair processes accompanied by increased cell proliferation and apoptosis have not been connected to stone-forming processes in tissues where calcifications are present. Thus, calcifications present in stone-forming tissues can serve as a clinical histopathological model for studying the process of stone formation and its effects on the surround tissues. Particularly large depositions of calcium salts lead to the formation of salivary gland stones (sialoliths) and kidney stones. The extent to which such a calcium stone obstructs the duct determines the extent of gland changes due to the stone and thus its link to the cause and effect relation of the processes of inflammation and/or fibrosis.

Sialolithiasis is a major cause of salivary gland dysfunction, and the mechanisms that lead to stone development are still unclear. Extraskelatal calcification can be a result of inflammation, however the reverse is true as well, as calcium deposits can trigger an inflammatory response. There are evidences to suggest a link between calcification and salivary duct carcinoma (González-Arriagada, Santos-Silva, Ito, Vargas, & Lopes, 2011) and ductal carcinoma of the breast (Hayes, Brodie, O'Doherty, & Quinn, 2013). In this manuscript, we present a new association among microcalcifications and cell injury-repair process, apoptosis, and cell proliferation in salivary glands with ductal stones as a clinical condition. The purpose of this study was to investigate such an association and its clinical significance by a comprehensive histopathological analysis of tissue sections of patients with and without salivary gland stones, or sialolithiasis. We propose a link between ectopic calcifications in the submandibular glands (SMGs) of these patients with related phenomena such as inflammation, fibrosis, and cell proliferation.

2. MATERIALS AND METHODS

2.1 Tissue preparation

Specimens were formalin-fixed, paraffin-embedded (FFPE), de-identified (not individually identifiable to any person) tissue sections from a Georgetown University tissue bank through an exempt Institutional Review Board (IRB) protocol (protocol number: 2010-423; PI: Bandyopadhyay). These were archival specimens of four human SMGs that had been excised because of irretrievable sialoliths ("stone glands") and four normal SMGs removed during neck dissections for head and neck cancers ("non-stone glands"). Two of the four stone glands are from males and two from females and the ages ranged from 34–52 years old. Similarity, among the four non-stone glands, two are from males and two from females, and the age ranged from 32–52 years old. To determine the extent of damage adjacent to the

stone compared to the distal and associated histopathological signatures, we have used only those samples where the entire gland was available. This criterion resulted in a smaller sample size. The sections were cut at 6 μ m from near mid-gland in order to sample as much of the gland as possible in one section, including the main excretory duct. Deparaffinized sections were subjected to stains and immunohistochemistry as follows.

2.2 Stains

Hematoxylin and eosin (H&E) was used for overall histologic assessment and to delineate inflammation and fibrosis. Sections were stained with the protocol for, and ready-to-use solutions obtained from, Richard-Allan Scientific, Kalamazoo, MI.

Alizarin Red S was used to visualize calcium deposits. Alizarin red S stains calcium specifically, reacting with calcium phosphate at pH 7 and pH 4.2 and reacting with calcium oxalate at pH 7, but not at pH 4.2. (Lombaert et al., 2008). Sections were washed in PBS and stained with 2% alizarin red S (A5533, Sigma Chemical Company, St Louis, MO, USA), rinsed in 0.003% in 1% KOH in H₂O (pH was adjusted to pH 4.3 with 0.5% NH₄OH) for 30 minutes at room temperature.

Masson's trichrome method for connective tissue is a triple stain that was used primarily to facilitate delineation of areas of fibrosis; staining was carried out employing a Masson's Trichrome Stain Kit, Artisan™ (AR173) in an Artisan™ LinkPro Special Staining System autostainer (Dako, Carpinteria, CA). The three stains are Weigert's iron haematoxylin (nuclei, black), aniline blue (collagen fibers, blue) and Biebrich scarlet-acid fuchsin (all other tissue components, pink or red).

2.3 Immunohistochemistry (IHC)

Proliferative activity—Cycling cell nuclei were labeled with monoclonal mouse anti-human antibodies to Ki-67 (Clone MIB-1, Agilent Technologies, Santa Clara, CA) (Bai et al., 2013).

Apoptosis—The cytoplasm of cells undergoing programmed cell death was labeled with monoclonal mouse anti-human antibodies to Caspase-3 (Caspase-3 E-8, sc7272, Santa Cruz Biotechnology, Santa Cruz, CA) (Bai et al., 2013).

IHC Procedures—Antibodies were localized as a brown precipitate via reaction with H₂O₂ and 3, 3 diaminobenzidine (DAB), using Envision and dual link HRP in a Dako (Carpinteria, CA) autostainer, and counterstained with hematoxylin. Native peroxidase was quenched in 3% H₂O₂. Reactions were enhanced by antigen retrieval in a pH buffer solution in a water bath. Reaction for Caspase-3, but not Ki-67, was enhanced using a mouse linker to amplify the signal of the primary mouse antibody. Positive controls for both Ki-67 and Caspase-3 were sections of human tonsil. For negative controls, the primary antibody was replaced with a negative control cocktail of mouse IgG1, IgG2a, IgG2b, IgG3 and IgM.

2.4 Image Quantification with ImageJ

Using digital photomicrographs of the slides obtained from scanning in Aperio ImageScope, areas of calcification in the Alizarin Red-stained slides, inflammation and fibrosis in the H&E and fibrosis in Masson's Trichrome slides, and the entire section, were quantified using the software ImageJ (NIH). In order to reduce the element of bias, images of the photomicrographs were converted to an 8-bit black and gray image. This was performed in order to visibly see the areas on the image of the photomicrographs where calcification has occurred. The program (Image J) then summates the darkest areas of the photomicrograph images, which coincide, with the areas of calcification. Based on these calculations, the total areas of calcification, inflammation, and fibrosis per section in salivary gland stone patients and non-salivary gland stone patients were calculated. In addition, the percent areas of inflammation and fibrosis per section in non-stone glands and areas proximal and distal to the stone in stone glands were calculated with photomicrographs of the H&E-stained sections.

2.5 Proliferation and Apoptosis

Ki-67- and Caspase-3-labeled and unlabeled cells were counted by cell type (acinar and intercalated, striated, and main and other excretory ducts) in the entire histologic section of each gland under 400X magnification using a Laboratory Counter (Clay-Adams, Parsippany, NJ). The percent positive cells (*labeling index*) of each cell type for each gland was computed by dividing the reacting cells by the total (reacting + non-reacting) cells in the section. Then the mean percent positive cells $\pm$ standard error (SE) was computed for each antibody and cell type in the stone and non-stone glands.

2.6 Statistical Analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM). Variance among three or more groups was analyzed by ANOVA. If this was significant, independent t-tests were then done to determine which groups showed a significant difference from one another. $P < 0.05$ was considered to be statistically significant.

3. RESULTS

3.1 Overview

Non-stone glands had only two to four small periductal foci of lymphocytes per section (Fig. 1a). Dense collagen fibers accompanied and were restricted to the immediate vicinity of the larger ducts and blood vessels in the stroma of normal glands, serving as part of the supporting structure of the gland (Fig. 3a and b). Mucous ("goblet") and ciliated cells were uncommon in the main and other large excretory ducts (Fig. 1b). The stone glands showed an uneven distribution of inflammation and fibrosis. In three stone glands, many lobules had inflammation with mild to no fibrosis, others, moderate to severe fibrosis with mild inflammation (Fig. 1d and 3d), and still others, little or no fibrosis or inflammation. In lobules with inflammation, fibrosis, or both, the acini were reduced in number and many had atrophied with only duct-like structures remaining (Fig. 1c, 1d, 3c, and 3d). A feature of note was the hyperplasia of mucous ("goblet") and ciliated cells (Fig. 1e) and stratified

squamous metaplasia in the main excretory and a few other large excretory ducts. One stone gland had no inflammation, fibrosis, or hyperplasia or metaplasia of the large excretory ducts, i.e., was indistinguishable from the non-stone glands in these regards. Two stone glands, but no non-stone glands, had one or more collections of oncocytes (Fig. 1f), which may represent oncoytosis, an occasional incidental finding in salivary glands from older individuals (Chang & Harawi, 1992).

3.2 Inflammation and Fibrosis

The mean areas of inflammation and collagen in the stone and non-stone glands, as determined by the ImageJ method with H&E stained sections, are depicted in Figure 2, and of collagen with the Masson-stained sections, in Figure 3. The non-stone glands exhibited very few inflammatory cells and no fibrosis. Therefore, the areas of inflammation and fibrosis in the stone glands are defined as the differences between the non-stone and stone glands.

Overall, stone glands displayed a greater area of fibrosis, but not inflammation, than non-stone glands (Fig. 2 and 3). Areas in stone glands proximal to the stone had a greater area of fibrosis than areas in stone glands distal to the stone (Fig. 2).

3.3 Calcification

Non-stone glands had almost no foci of calcification (Fig. 4a). Stone glands displayed focal calcifications around the acini, the walls of ducts, and the walls of blood vessels (Fig. 4b–d). Small stones were also observed obstructing ducts (Fig. 4e and F). Focal calcifications were in some cases accompanied by acinar atrophy, inflammation, and fibrosis (Fig. 4f). Quantification of the areas of calcification revealed that the stone glands displayed a greater area of calcification than non-stone glands (Fig. 5).

3.4 Cell Proliferation and Apoptosis

Proliferating (Ki-67-labeled) cells and cells undergoing apoptosis (Caspase-3-labeled) are illustrated in representative photomicrographs and summarized as mean percent labeled cells by cell type and gland in Table 1.

Non-stone glands displayed very few proliferating (Ki-67 labeled) cells (Fig. 6a and b). Stone glands exhibited somewhat more numerous proliferating cells in clusters or “hot spots” among the acini, striated ducts, and intercalated ducts, (Fig. 6c and d) Proliferating cells were strikingly numerous in the larger excretory ducts of stone glands, especially where hyperplasia of goblet and ciliated cells and stratified squamous metaplasia had occurred (Fig. 6e). The only statistically significant stone gland-non stone gland difference was the higher labeling index of Ki-67- labeled cells in the main duct of stone glands (Table 1).

Apoptotic (Caspase-3 labeled) cells were uncommon to rare in all cell types in non-stone glands (Fig. 7a and b). In stone glands, apoptotic cells were rare in the acini and uncommon in the intercalated and striated ducts and the smaller other excretory ducts (Fig. 7c–e), and numerous in clusters in segments of stratified squamous metaplasia in the main excretory

ducts. Apoptotic cells made up the vast majority of cells above the basal cell layer in the main and a few segments of larger other excretory ducts of stone glands where hyperplasia of goblet and ciliated cells had occurred (Fig. 7f). The only statistically significant stone gland-non stone gland difference was the higher labeling index of Caspase-3 labeled cells in the main duct of stone glands (Table 1).

4. DISCUSSION

The overall effect of the blockage of the SMG duct was the progressive but uneven degeneration of the gland. The areas of degeneration were characterized by varying degrees of inflammation, calcification, acinar atrophy, and fibrosis. The paucity of ciliated and goblet cells in the main ducts of the non-stone glands and their hyperplasia in the stone glands is consistent with previously reported findings in normal (Testa-Riva, Puxeddu, Riva, & Diaz, 1981) and obstructed (Testa-Riva, Riva, & Puxeddu, 1987) human salivary glands.

Glandular microenvironment can be altered in chronic submandibular and parotid sialadenitis where microliths are found in stroma, particularly around intercalary ducts, in lumina and in parenchyma, and contained calcium crystals (Triantafyllou, Harrison, & Donath, 1998). Cellular debris due to luminal microliths are possibly formed as a result of stagnation of secretory material rich in calcium and membranous debris (Harrison, Triantafyllou, Baldwin, & Schäfer, 1993). Our observation may link such association between chronic sialadenitis in salivary gland microenvironment in stone condition. Interestingly, studies have shown in rats that melamine-related kidney stone formation involved inflammation, crystal-induced injury of epithelial cells, and apoptosis. MCP-1, which helps to induce inflammation, was upregulated in rats with kidney stones (Lu et al., 2012). It is likely that salivary gland stone formation similarly provides a stimulus for inflammation. Since other studies have demonstrated that inflammation induces calcification (Fukuyo et al., 2014; Watson et al., 1994), it is likely that the inflammation induced by salivary gland stones induces calcification in salivary gland tissue. The inflammation and calcification occurring in the salivary gland would then reinforce each other in a positive feedback loop.

Additionally, the injury of epithelial cells led to their apoptosis (Lu et al., 2012). These apoptotic cells then provided a substrate for aggregation and attachment of melamine-related crystals, thus allowing for the growth of the calcium stone. It is also well established that injured epithelial cells are a substrate for the aggregation and growth of calcium stones (Jia et al., 2014; de Water et al., 1999). Thus, it is likely that surface cell injury and apoptosis contributes to salivary gland stone formation. It is also clear that the inflammatory response is a known cause for fibrosis (Stramer, Mori, & Martin, 2007). Boonla et al. (Boonla et al., 2014) reported that S100A8, an inflammation marker, and fibronectin, a fibrosis marker, were both upregulated in the kidneys during the occurrence of kidney stones. Thus, it is likely that the fibrosis occurring in the SMG is the result of inflammation induced by the salivary gland stone in response to tissue damage by calcium crystals.

The overall effect of ductal blockage by salivary gland stones is parallel to what has been observed with ligation of the ducts in experimental animals. Maria et al. (2013) reported that

double ligation of Stenson's duct of the rabbit parotid gland led to a sequence of inflammation of the lobules, atrophy and apoptosis of acinar cells, and cell proliferation which occurred almost exclusively in the largest excretory ducts. These changes were the result of the build-up of back pressure caused by the double ligation of the duct, which led to an accumulation of secretory material in dilated ducts and acini. Sixty days after ligation, a steady-state was reached in which there was little or no salivary secretion because of the reduced number and atrophy of the acini, inflammation was uncommon, and proliferation and apoptosis were rare.

The similarities between what was observed in human salivary gland stone patients and ligation models of salivary gland ducts validate the ligation model for studying the effects of ductal blockage on salivary glands. The inflammation, fibrosis, and acinar atrophy observed in the SMG tissue from stone patients could be attributed to the build-up of back pressure from ductal blockage. However, unlike the rabbit parotid gland at 60 days post-ligation, not all lobules were at the same stage in the human stone glands. The build-up of back pressure may also be relevant to the high concentration of proliferating cells in large excretory ducts, as the resulting dilation would require the cells to proliferate to maintain ductal integrity.

There have been a number of studies demonstrating a link between calcification and salivary duct carcinoma (SDC) and ductal carcinoma *in situ* (DCIS) of the breast (Weon et al., 2012; Hayes, Brodie, O'Doherty, & Quinn, 2013; Morgan, Cook, & McCarthy, 2005). Notably, DCIS is histologically similar to SDC. Weon et al. (Weon et al., 2012) reported that half of the malignant SDC in their study displayed calcification. Morgan et al. (Morgan, Cook, & McCarthy, 2005) reported that 30 to 50% of all breast cancer cases displayed microcalcifications. Additionally, there have been a number of studies demonstrating the presence of molecular markers associated with calcification in DCIS, prostate cancer, and submandibular gland tumors (Mandavilli, Singh, & Sahmoun, 2013; Wang et al., 2010; Wykoff et al., 2001; Coussens & Werb, 2002; Takai, Hyun, Murase, Hosaka, & Mori, 1984). These molecular markers include carbonic anhydrase (CA) IV and VI, TRPM7, and TRPC1 in DCIS and TRPC6 in prostate cancer. Finally, both inflammation and fibrosis have been linked to an increased risk for cancer (Coussens & Werb, 2002; O'Connor & Gomez, 2014).

Therefore, salivary gland stones and the calcification of salivary gland tissue could increase the risk of SDC by inducing inflammation and fibrosis. It is noteworthy that atrophy of acini and granular ducts to duct-like structures followed by metaplasia to squamous epithelium were early steps in the 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced carcinomas in rodent submandibular glands (Kim, Spencer, Weatherbee, & Nasjjleti, 1974; Takai, Hyun, Murase, Hosaka, & Mori, 1984). An abnormally high rate of turnover (numerous proliferating and apoptotic cells), as observed in the larger excretory ducts of stone glands especially where ciliated and goblet cell hyperplasia and squamous metaplasia had occurred, increases the susceptibility of cells to mutations which may cause cell proliferation to be deregulated. Deregulation of cell proliferation could then potentially lead to dysplasia. The larger ducts, but not acini or small ducts, are labeled with two or more putative markers of "stem" (more accurately, progenitor), cells in mature salivary glands (Lombaert et al., 2008). It seems plausible to suggest that increased proliferation in these cells would be even more apt to lead to cancer. It is of interest in this regard that hyperplasia of ciliated and goblet

cells has been reported in a salivary gland tumor of the palate (Guccion, Redman, Calhoun, & Saini, 1997). However, in order to establish a causative relationship between salivary gland stone and calcification, inflammation, and fibrosis, the expression of molecular markers for the respective changes will need to be studied. It would also be interesting to determine whether a link could be established between salivary gland stone and SDC.

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List of Abbreviations

CaP	Calcium phosphate
SMG	Submandibular gland
FFPE	Formalin fixed paraffin embedded
H&E	Hematoxylin and eosin
DAB	3,3 diaminobenzidine
CA	Carbonic anhydrase
CMBA	9,10-dimethyl-1,2-benzanthracene

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Highlights

- Calcification in sialolithiasis, ductal obstruction.
- Clinical pathology and predisposition in patients with sialolithiasis.
- Interrelationship – Calcification, Inflammation and fibrosis, and stone pathology.
- Calcification, apoptosis, cell proliferation, salivary gland cancer.

Figure 1.

General histologic features. A, B, Normal glands; C–F, Stone glands. A. Serous acini are almost filled with secretory granules (purple dots). There is a focus of periductal infiltration of lymphocytes (arrow). B. This segment of main excretory duct has a solitary mucous (“goblet”) cell (arrow). C. Lobule with atrophic (few, if any, secretory granules) acini and a moderate to heavy infiltration of inflammatory cells, mostly lymphocytes and plasma cells. D. Advanced stage lobular atrophy, in which acini appear to be absent and ducts and duct-like structures are sparsely distributed in a fibrotic (pink collagen fibers) stroma. E. The epithelium of this large excretory duct shows extensive hyperplasia of goblet cells (arrow)

and ciliated columnar (double arrow) cells. There is a moderate collection of lymphocytes and plasma cells in the surrounding stroma. F. Oxyphilic (oncocytic) metaplasia. Some of the oxyphilic cells (deep pink cytoplasm), in this nodule retain the general structure of striated ducts. a = serous acini; f = fat (adipose) cells; m = mucous acini; sd = striated ducts. Hematoxylin and eosin stains. Magnification bars: A, C = 150 μm ; D–F = 100 μm ; B = 50 μm .

Figure 2.

Quantification of H&E results (T-bars at top of solid bars) represent SEM. The differences in the areas of inflammation among the three groups were not statistically significant, whereas differences in the areas of fibrosis among the three groups were significant ($P > 0.05$ and < 0.05 , respectively, by one-way ANOVA). Independent t-tests were then done to determine which groups showed a significant difference in fibrosis from one another. Areas proximal to the stone (gland hilus) had a greater area of fibrosis than a similar hilar area of non-stone patients (*, $P < 0.05$), but other differences were not significant.

Figure 3.

Photomicrographs of human submandibular glands, Masson's Trichrome stain. A. Normal gland. Thick bands of collagen (blue fibers) are limited to the inter-lobular septa. Ducts and plump acini fill the lobules and intralobular collagen fibers are thin and delicate. B–D. Stone glands. B. Overview showing interlobular variation in the extent of acinar atrophy, inflammation, and intra-lobular fibrosis. C. Lobules with mild acinar atrophy and a moderate infiltration of mononuclear cells. D. Lobule with a few large ducts, scattered duct-like structures, strophic mucous acini, no serous acini and intra-lobular fibrosis. a = serous acini; m = mucous acini; sd = striated ducts. Magnification bars: A, B = 150 μm ; C, D = 100 μm .

Quantification of Masson's Trichrome results. Stone glands showed a significantly greater area of fibrosis (*, $P < 0.05$ by independent t-test) than in non-stone glands, but this was not the case for inflammation.

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Figure 4.

Alizarin Red stain. A, normal gland; B–F, stone glands. Some acini have calcium-rich secretory granules (weak red stain; arrow). B. Deep red marks focal calcifications among acini and ducts. C. The walls of a striated (sd) and intercalated (id) duct have focal calcifications. Nearby serous (a) and mucous acini are intact. D. The walls of this blood vessel (v) are ringed with calcification. E. The lumen of one of the striated ducts is obstructed by a stone. F. Two lobules have atrophic acini (a) surrounded by inflammation, and there is a large area of fibrosis (f) around some larger ducts. There are small stones in two ducts. Magnification bars = 150 μ m (A, D–F) and 100 μ m (C).

Figure 5. Quantification of calcifications (Alizarin Red stained). Stone glands showed significantly greater total area of calcification in SMG than non-stone glands, (*, $P < 0.05$ by Independent t-test).

Figure 6.

Human submandibular glands proliferative activity (Ki-67 immunohistochemistry). A, B. Normal glands. Ki-67-positive cells (brown nuclear stain) were generally uncommon in the stroma and parenchyma, but several per 100× field occurred in widely scattered clusters. One such cluster is illustrated in A. The main excretory duct in B has none. C–F. Stone glands. C, D. Lobules with acinar atrophy and inflammation. Ki-67-positive cells are numerous in intercalated and striated ducts, duct-like structures, and atrophic acini. E. Ki-67-positive cells are very numerous in the basally located cells of this large excretory duct with hyperplasia of ciliated columnar and goblet cells. F. Main excretory duct with

abrupt transition (arrow) from hyperplastic columnar and goblet cells to metaplastic stratified squamous epithelium. The majority of the basally located cells of the latter are Ki-67-positive. Ki-67-positive cells also scattered in the foci and bands of mononuclear inflammatory cells in the connective tissue around the excretory ducts. a = serous acini; id, sd, ed = intercalated, striated and excretory ducts, respectively. Hematoxylin counterstain. Magnification bars: A, B, C, E, F = 100 μ m, D = 50 μ m.

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Figure 7.

Human submandibular glands (caspase immunohistochemistry). A, B. Normal glands.

Caspase-positive cells (cytoplasmic reaction) were generally uncommon in the stroma and parenchyma. Two are illustrated in a striated duct in B. C–F. Stone glands. Caspase-positive cells including lymphocytes were common in the stroma (arrows), focally numerous in the intercalated and striated (D) ducts and the luminal cells of the smaller excretory (E) ducts, and rare in the basal cells of the excretory ducts (D, E). Among the hyperplastic goblet and ciliated cells in the main excretory ducts (F), caspase-positive cells were common and all of the others had weak to moderate brown staining, Caspase-positive acinar and myoepithelial

cells were not seen in either the normal or stone glands. a = serous acini; id, sd, ed = intercalated, striated and excretory ducts, respectively; m = mucous acini. Hematoxylin counterstain. Magnification bars: A, F = 100 μ m; B–E = 50 μ m.

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Mean Labeling Indices $\pm$ SEM by Immunohistochemical Reactions to Ki-67 and Caspase-3 Antibodies

Table 1

Gland	(n)	Antibody	Main ED	Other ED	SD	ID	AC
Stone	(4)	Ki-67	15.47 $\pm$ 6.00 *	3.61 $\pm$ 2.08	1.18 $\pm$ 0.78	1.85 $\pm$ 0.82	0.37 $\pm$ 0.25
Non-Stone	(4)	Ki-67	1.88 $\pm$ 1.07	0.49 $\pm$ 0.18	0.29 $\pm$ 0.15	0.64 $\pm$ 0.18	0.19 $\pm$ 0.1
Stone	(4)	Caspase-3	37.24 $\pm$ 15.37 *	2.38 $\pm$ 0.91	1.30 $\pm$ 0.47	1.40 $\pm$ 1.28	0.08 $\pm$ 0.07
Non-Stone	(4)	Caspase-3	1.16 $\pm$ 1.23	0.59 $\pm$ 0.39	0.48 $\pm$ 0.38	0.00 $\pm$ 0.00	0.01 $\pm$ 0.01

* Stone gland value is significantly greater than non-stone gland value ($p < 0.05$).
AC= acini (serous and mucous); ID, SD, ED = intercalated, striated and excretory ducts, respectively.