A Model to Study Gland Regeneration/Development in Rat: The Expression of Metalloproteinase- 9 and Extracellular Matrix Proteins

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Summary

A model to study gland regeneration as a similar phenomenon to gland development is proposed. This study evaluated the expression of metalloproteinases (MMPs), laminin (LN) and type I and III collagen in the regeneration of the rat submandibular gland (SMG). Eighteen 30-day-old Wistar male rats were anesthetized, and the lower third of their SMG left lobe was excised. The animals were killed on the 2nd, 3rd, 7th and 15th postoperative days, and their SMG lobes were removed, fixed and processed in paraffin. Immunohistochemistry was used to label type I and III collagen, laminin, and MMP-9. The avidin-biotin technique was used, and the reaction was developed with diaminobenzidine. On the 2nd day, MMP9 expression was intense in the margins of the regenerating area and inside striated duct cells in the preserved gland. On the 3rd day, duct cytoplasm labeling persisted and was more intense than in the surrounding mesenchyme, where labeling increased along time. Concurrently, LN labeling in the basal lamina of epithelial buds was intense and discontinuous. Types I and III collagen were present during the whole process, which showed their importance for the regeneration process. The behavior of extracellular macromolecules observed in this study is similar to their behavior in gland development.

Introduction

Saliva is a complex fluid that wets the oral cavity and plays an important role in the maintenance of good general health. An adequate salivary flow is fundamental for normal physiological functioning. The integrity of the salivary gland is a requisite for the synthesis and secretion of salivary fluid.

Previous studies performed in our laboratory showed that when a partial excision of the rat submandibular gland (SMG) is accomplished, the regeneration phenomenon starts early in the process and this is similar morphologically to embryogenesis (*Fossati et al., 2004*). Therefore, it is supposed that if gland regeneration is dependent on those same factors that affect SMG embryogenesis, this method could be used as a model to study it.

The fundamental role of the extracellular matrix (ECM) molecules, which form the glandular stroma, in the branching morphogenesis and cytodifferentiation of the salivary gland during embryonic development has been well established in the literature (*Cutler, 1990; Spooner & Hardman, 1992; Fossati, 2000; Kumagai & Sato, 2003; Furuse et al., 2004*).

Collagen, one of the several ECM components, is found during all embryological development and triggers cytoplasmic signaling pathways, which confirms its importance in the development of the salivary gland (*Grobstein & Cohen 1965; Cutler, 1990; Fossati, 2000; Kumagai & Sato, 2003*).

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Laminin (LN), another ECM glycoprotein, is expressed exclusively in the basal lamina (BL), an extracellular structure located between epithelial and connective tissues. The BL plays an essential role in the epithelial-mesenchymal interaction, as it acts as a molecule filter and promotes organ growth. The expression of the 15 laminin isoforms is tissue-specific embryonic-stage dependent (*Aumailley, 1995; Gumbiner, 1996; Kadoya & Yamashina, 2005; Kleinman & Martin, 2005*).

Metalloproteinases (MMPs) are proteolytic enzymes secreted locally by cells. They degrade ECM components, such as collagen and laminin, and promote the turnover necessary for remodeling, wound healing, and organogenesis (Aumailley, 1995; Gumbiner, 1996). They are regulated with precision under normal physiological conditions. The balance between MMPs and their tissue inhibitor of metalloproteinase (TIMPs) is critical for ECM remodeling **(***Nagase et al., 2006*).

This study investigated the behavior of metalloproteinases and the expression of ECM molecules – type I and type III collagen and laminin - at different stages of submandibular gland (SMG) regeneration in rats submitted to partial removal of a gland lobe in order to establish the similarity of gland embryogenesis and regeneration process.

Materials and Methods

Animals

Eighteen Wistar male rats were obtained from the colonies of CREAL of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. They were 30 days old weighing 250-300g. Standard pelleted (Nutrival, CR-1 - Nutrival Nutrientes – Curitiba - Brazil) and water was provided *ad libitum* to all groups. Sterilized bedding (Vet-Sul, Porto Alegre, Brazil) and proper ventilation with temperature range between 20-22^{o} C was provided. The rats were exposed to proper light and dark cycle (12 h each of light and darkness). Sterilized plastic cages (Beira Mar - São Paulo, Brazil) with a steel cover housed three animals each during the experiment

All procedures received the relevant clearance from the local Ethics Committee for Animal Care and Use in Experiments.

Surgical Procedures

The animals were anesthetized with ketamine (Ketalar, Parke-Davis, São Paulo, Brazil) 30mg/100g IM and xylazine (Rompum, Bayer S.A., São Paulo, Brazil) 2mg/100g IM, and the lower third of the SMG left lobe was excised. After the operation, food and water were provided *ad libitum* at night. On the 2nd, 3rd, 7th and 15th postoperative days, animals were killed and both SMG lobes were removed.

Laboratory Procedures

The material collected was fixed in 30% Methacarn solution, and paraffin (Biotec®, Porto Alegre, Brazil) embedded. After that, 5-**µ**m sections were obtained and mounted on silane-coated glass slides, which were stained using one of the following methods:

One slide of each group was randomly chosen and stained using the hematoxylin-eosin (Pró-Cito®, Porto Alegre, Brazil) technique.

Peroxidase immunohistochemistry was used to label the type I and III collagens, laminin, and MMP-9. The sections were deparaffinized and rehydrated in graded alcohols, and endogenous peroxidase activity was blocked using 30 vol H_2O_2 and methanol (1:1) for 10 minutes. Normal serum from a secondary antibody host was used to block nonspecific sites. Polyclonal primary antibodies were purchased from Chemicon (anti-FN, anti-collagen I and III and MMP 9; **Temecula, California, USA**) and Sigma (LN; Sigma-Aldrich, St. Louis, USA). The LN antibody recognized some protein isoforms. All sections were incubated overnight in a humidity chamber at 4º C with primary antibody diluted 1:100 (PB + 0.2% Triton) and incubated for 2 h at room temperature with biotinylated goat anti-rabbit IgG secondary antibody, diluted 1:200. The avidin-biotin immunostaining technique was used, and the reaction was developed with diaminobenzidine. A negative control, which consisted of primary antibody omission, was included in all reactions.

The slides were analyzed under light microscopy

using an Olympus CX microscope, and photographed using a digital camera (Sony Cyber-Shot P10, Oklahoma, Japan) by an experienced histologist (accomplishes the analysis). Identification labels of groups were covered in order to guarantee "blind" examination and description of microscopic images.

Results

In the early regenerative stage, a stalk-extensive proliferation of epithelial cells from the preserved gland to the excised region was found on the 2nd postoperative day (fig.1A, 2A, 3A).

Type I collagen expression was observed in all stages. On the 2nd day of regeneration, a small amount of this protein was found in the stroma, and a greater label intensity was observed in the cytoplasm of epithelial cells of the stalk, the newly-forming duct and in some mesenchymal cells. On the 3rd postoperative day, greater immunolabeling was observed in the margins of the regeneration area, and a cytoplasmic protein pool was still seen in the ducts and newly-forming secretory endings. As the process progressed, connective expression increased, and a concentration of type I collagen was seen in the basal lamina (BL), surrounding structures close to the regenerated area (Fig.1A, B, C). The aspect on the 15th day was similar to that of the preserved left lobe and the right intact lobe, and the protein was seen among the acini and the ducts (data not shown).

Type III collagen labeling was greater on the 2nd postoperative day; it seemed to surround the stalk and be distributed around the whole epithelial cord. On the 3rd day, it was concentrated in the BL of the epithelial structures on the proximal area of the wound. This distribution became homogeneous on the 7th day, when the protein was spread through the entire stroma and expressed mainly in front of the new glandular structures (fig. 2).

Laminin (LN) is another ECM glycoprotein and a molecule found only in the BL. On the 2nd regeneration day, a continuous distribution of LN was concentrated in the BL of epithelial buds that

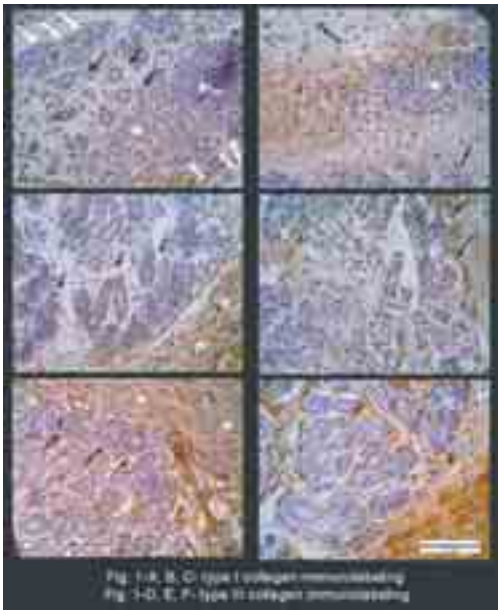

Figure 1. A) Regeneration front (white arrows)- Type I collagen immunolabeling on the 2nd postoperative day - slight protein labeling in stroma, inside stalk (asterisks) and ductal cytoplasm (black arrows), and in some mesenchymal cells. B) Regeneration area- Type I collagen immunolabeling on the.3rd postoperative day - greater stroma concentration on regeneration front (asterisks) and cytoplasmic labeling of prospective ducts and secretory endings located near excised area (black arrows). C) Regeneration front- Type I collagen immunolabeling on the 7th postoperative day - intense protein labeling in glandular stroma (asterisks); also concentrated in BL of structures located in wound margin (arrows). D) Type III collagen immunolabeling on the 2nd postoperative day- fibrillar protein network surrounding entire stalk (asterisk); also seen in some cytoplasmic mesenchymal cells (black arrows). E) Type III collagen immunolabeling on the 3rd postoperative day - protein concentrated around epithelial structures (black arrows) in front of wound (asterisk). F) Regeneration area- Type III collagen immunolabeling on the 7th postoperative day - protein distributed in entire glandular stroma, which gives gland more homogenous aspect.

Diaminobenzidin staining and Hematoxilin counter staining

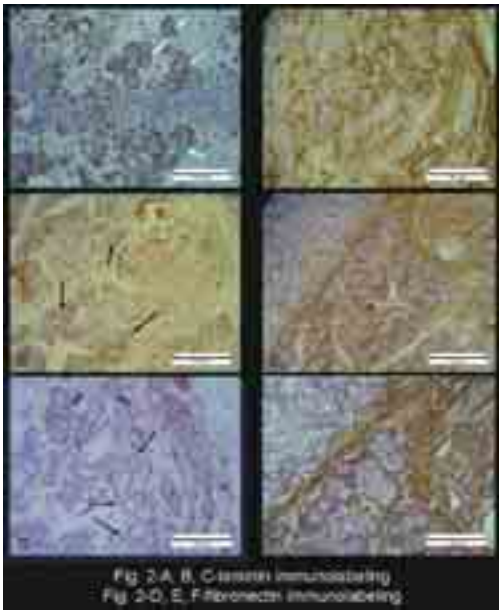

Figure 2. A) Regeneration front- Laminin immunolabeling on the 2nd postoperative day - epithelial rudiments "sprout" from epithelial stalk; with heavy protein labeling of BL (arrows). B) Regeneration area- Laminin immunolabeling on the 3rd postoperative day – same aspect persists; great discontinuity of labeling around some epithelial structures (arrows). C) Regeneration area- Laminin immunolabeling on the 7th postoperative day – regenerated zone with structures similar to ducts (arrows); persistent discontinuity of laminin labeling. D) Regeneration area- Fibronectin immunolabeling on the 2nd postoperative day - intense fibronectin immunolabeling in stroma around ducts and epithelial bud rudiments. E) Fibronectin immunolabeling on the 3rd postoperative day - labeling heavier in regenerated region, which seems enclosed by fibronectin (asterisk). F) Regeneration area- Fibronectin immunolabeling on the 7th postoperative day - protein observed throughout connective tissue.

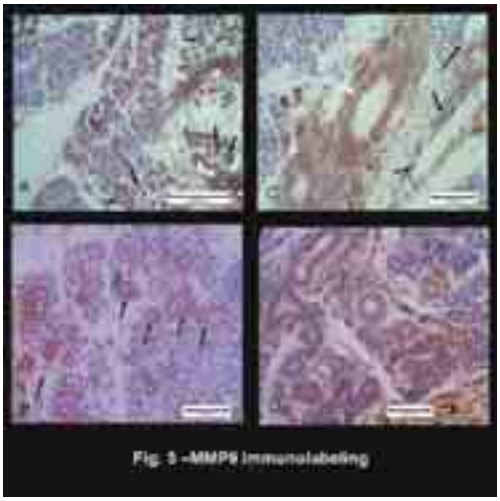

Fig. 3 - A) Regeneration area-MMP9 immunolabeling on the 2nd postoperative day - enzyme in regenerated front (white arrows) and inside cytoplasm of striated ducts near wound (black arrows). B) MMP9 immunolabeling on the 3rd postoperative day - same aspect persists, with labeling of cytoplasm heavier in ducts (black arrows) than in prospective secretory endings. C) Labeling of striated ducts cytoplasm (white arrows) and BL of the endothelium (black arrows).D) MMP9 immunolabeling on the 7th postoperative day - enzyme concentrated homogenously in stroma in front of wound (asterisk); slight immunostaining of cytoplasm of BL vessels, duct and acinus.

seemed to "sprout" from the stalk. On the 3rd day, LN was expressed around some gland rudiments and its distribution was discontinuous. On the 7th day, the regenerated area had various duct-like structures under differentiation, and protein labeling showed discontinuities in the BL. However, features found on the 15th day were similar to those of a normal gland.

In the early stages of the regenerative process, intense FN labeling was seen in the mesenchyme surrounding epithelial buds. The expression of this protein, necessary for cellular migration in

embryonic tissues, was observed in the excised region around gland rudiments that were starting to develop. On the 3rd postoperative day, there was intense labeling far from the regenerated area, which seemed to mark the margins of the wound. In later stages, FN distribution was similar to that of the proteins described above (fig. 4).

The margins of the regeneration area showed intense immunolabeling to the MMP9 proteolytic enzyme on the 2nd postoperative day. In the region of the preserved gland near the excised area, MMP9 was found inside the striated duct cells, concentrated in the BL of vessels walls and in the cytoplasm of stalk cells. On the 3rd day, labeling in the duct cytoplasm of structures close to the wound margin was more intense than in the surrounding mesenchyme. A small protein pool was seen in the cytoplasm of newly-forming secretory endings. As the process progressed, there was a decrease in duct cytoplasm expression and intense and homogenous stroma immunolabeling (fig. 5). On the last postoperative day under study, it was slight and circumscribed to stroma between ducts and acini.

Discussion

This study investigated the behavior of metalloproteinases and the expression of ECM molecules – type I and type III collagen and laminin - at different stages of submandibular gland (SMG) regeneration in rats submitted to partial removal of a gland lobe. Our findings provide evidence that the behavior of extracellular macromolecules observed in regeneration is similar to behavior during gland development.

Data in our study suggest that MMP9 plays a fundamental role in glandular regeneration, and that it is primarily produced by striated duct cells. In the early stages of regeneration, MMP9 expression was intense at the wound margins, as well as in the cytoplasm of some stalk cells. At that time point, marked cytoplasmic duct labeling was observed. Labeling decreased as the process progressed, and protein expression moved to the mesenchyme. Their slight immunolabeling on the 15th postoperative day seems to indicate little enzyme expression that may enable normal tissue turnover. MMPs play a central role in wound healing, tissue repair and morphogenesis because they promote ECM digestion, which is necessary for cell migration. These findings are in agreement with results reported by Legrand et al. (*1999*), who studied cellular migration in wound healing in the respiratory epithelium. They found remodeling of a time-provisional secreted ECM under MMP9 control, which enabled the cellular migration necessary for wound healing. in the same way, in our study this glycoprotein was also found in BL of vessel walls, probably supporting leukocyte diapedesis from the inner vessels to the regenerated area. This finding may help to confirm the role of MMP9 in "tissue bed" preparation for the regeneration process

Analyzing expression of Laminin in our study, it was observed that labeling was more intense close to the regeneration area than in the preserved gland in the same lobe. Its distribution was discontinuous in the BL of epithelial rudiments, especially in the early stages of regeneration. The BL discontinuity was more evident on the 3rd day, which may indicate a turnover necessary for the formation of the SMG morphogenetic pattern. A similar discontinuous aspect has been observed by other authors. In agreement with our results, those authors studied SMG development and found that the BL became thinner in those places where the epithelial rudiments began to grow (*Cutler & Chaundry, 1973; Kadoya & Yamashina, 1999; Fossati, 2000*).

In a later stage (7th day), duct-like structures showed accentuated proliferation and already had a central lumen, although little differentiation was seen. At this time point, some structures showed discontinuity of BL molecules. This distribution seems to be necessary for molecule transit, which promotes full organ differentiation. On the 15th day, LN labeling in the regeneration area was concentrated around acini, ducts and vessels, similarly to the labeling of the preserved portions of the gland. Therefore, protein labeling seems to be discontinuous only in the regenerative stages when ducts and acinus-

branching morphogenesis may be under way.

In our experiment, type I and type III collagens were seen during the whole process, and their expression was greater in the regeneration area.

On the 2nd postoperative day, type I collagen was found in the gland stroma and in the cytoplasm of epithelial rudiments and mesenchymal cells, which suggests their synthesis by both tissues. On the 3rd day, greater protein expression was observed, probably because of greater stroma concentration. Fossati et al (*2004*) have recently investigated rat SMG regeneration using the H/E staining technique, and found connective tissue accumulation on the regenerative area. In the right lobe and in the preserved left area, the type I collagen was found outside the cells, homogeneously distributed in the entire gland stroma. On the 7th day, type I collagen was distributed mainly in the margins of the regeneration area, and the cytoplasmic pool was decreasing. On the 15th day, distribution was similar to that observed in the preserved gland structure. The analysis of these data suggest that the expression of type I collagen is essential for regeneration apparently triggering intracytoplasmic signaling pathways leading to the formation of the SMG morphogenetic pattern (*Fukuda et al., 1988; Nakanishi et al., 1988; Cutler, 1990; Fossati, 2000; Fossati et al., 2004*).

On the 2nd postoperative day, type III collagen was slightly distributed in the glandular stroma and heavily concentrated around the stalk (fig. 5 A). The heavy expression may be related to the extension of the area of the future regenerated lobe. This finding is in agreement with those reported by Furuse *et al*. (*2004*), who studied immunoexpression of some ECM proteins in salivary glands of seven human fetuses at different gestational stages. They found a similar aspect that we have seen in the regeneration in the earliest stages of development: type III collagen was seen as fine fibers surrounding the glandular structures and allowing the extension of the future lobe.

On the 3rd day of our study, type III collagen was not only distributed in the stroma, but also very concentrated around some sprouting epithelial structures in the regenerated area. This suggests that, in the beginning of the regeneration process, type III collagen is concentrated in the BL of those structures undergoing morphogenetic branching. Some authors observed that, before SMG branching morphogenesis, type III collagen is concentrated inside clefts formed in the stalk, which stabilizes it and enables branching (*Nakanishi et al., 1986, 1988; Cutler, 1990*). Fossati (*2000*) suggested that type III collagen forms a reticular network that sustains the fast growth of the glandular parenchyma.

Moreover, type III collagen was also found in the cytoplasm of some parenchymatous and mesenchymal structures. On the 15th day, it was seen among epithelial structures as well as in their cytoplasm, exactly as observed both in the intact and in the preserved glands.

Conclusion

In conclusion, type I and type III collagens were found in all gland regeneration stages analyzed in this study, which demonstrates their importance for the regenerative process. The laminin discontinuity found in the early stages demonstrates the intense turnover that the BL undergoes during regeneration. The presence of MMP-9 seems to be associated with cellular migration, also necessary for regeneration. Therefore, gland regeneration seems to be dependent on those same factors that affect SMG embryogenesis.

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