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Controlling branching structure formation of the salivary gland by the degree of chitosan deacetylation

Ya-Chuan Hsiao [a,b,c], Chun-Nan Chen [a,d], You-Tzung Chen [d,e], Tsung-Lin Yang [a,f,*]

[a] Department of Otolaryngology, National Taiwan University Hospital and College of Medicine, Taipei, Taiwan
[b] Department of Ophthalmology, Zhongxing Branch, Taipei City Hospital, Taipei, Taiwan
[c] Department of Ophthalmology, College of Medicine, National Yang-Ming University, Taipei, Taiwan
[d] Graduate Institute of Clinical Medicine, National Taiwan University College of Medicine, Taipei, Taiwan
[e] Graduate Institute of Medical Genomics and Proteomics, National Taiwan University College of Medicine, Taipei, Taiwan
[f] Research Center for Developmental Biology and Regenerative Medicine, National Taiwan University, Taipei, Taiwan

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ABSTRACT

The salivary gland is characterized by ramified epithelial branches, a specific tissue structure responsible for saliva production and regulation. To regenerate the salivary gland function, it is important to establish the tissue structure. Chitosan is a deacetylated derivative of chitin with wide biomedical applications. Because of its deacetylated nature, chitosan has different properties when prepared with different degrees of deacetylation (DDA). However, the impact of chitosan DDA on the effect of regulating tissue structure formation remains unexplored. In this study, the embryonic murine submandibular gland (SMG) was used as a model to investigate the role of chitosan DDA in regulating tissue structure formation of the salivary gland. When chitin substrates with different DDA were used, the branching numbers of cultured SMG explants changed. Similar effects were observed in the culture with chitosan prepared using different degrees of acetylation. The mRNA expressions of type I and type III collagen were elevated in SMG explants with enhanced branching morphogenesis, as was the protein level. In addition to the amounts of collagen, type I and type III collagen fibers were spatially present in the epithelial–mesenchymal junction of developing branches in the culture with chitosan of a specific range of DDA. The branch-promoting effect of chitosan DDA was abolished when SMG explants were treated with collagenase, both early in the stage of branch initiation and with the establishment of the branching structure. The branch-promoting effect of chitosan DDA disappeared when antisense oligonucleotides were applied to specifically block type III collagen. This study demonstrates for the first time that DDA of chitosan affects tissue structure formation. The different proportions of side-chain components of chitin derivatives regulate structural formation of cultured SMG, indicating that DDA is an important parameter using chitosan as a biomaterial for tissue structure formation of the salivary glands.

1. Introduction

Many vital organs responsible for essential physiological functions are featured by their ramified structures. In mammals, these complex and intricate architectures are characterized by branching morphology. The specific tissue structures exist universally in the lung, the kidney, the pancreas and many excretory glands [1]. With this structure, the efficient transport system for gas, liquid and metabolite exchange can be established to meet the physiological demands of multicellular organisms. Ramified structures are generated by branching morphogenesis during organogenesis. Generally, a single bud originating from the epithelium initiates this branching process. It is followed by active and reciprocal interaction with surrounding tissue, during which the epithelial bud proliferates and ramifies to generate a tree-like architecture after numerous repetitive rounds [2].

The salivary gland regulates saliva production, which is essential for oral hygiene, food digestion and infection control [3]. When salivary gland function is impaired by disease or medical management, xerostomia occurs and adversely affects the health and function of the aerodigestric system. However, no effective treatment for xerostomia had been established until now [3]. To recover salivary gland function completely, regenerating the salivary gland may be an appealing approach. Because the salivary gland is an excretory organ characterized by ramified epithelial branches, establishing the tissue structure is important to salivary gland regeneration.

* Corresponding author at: Department of Otolaryngology, National Taiwan University Hospital and College of Medicine, Taipei, Taiwan. Tel.: +886 2 23123456x63526; fax: +886 2 23940049.
E-mail address: yangtl@ntu.edu.tw (T.-L. Yang).

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Chitosan, a biocompatible and biodegradable biomaterial, has many appealing biological properties such as antibacterial and antitumor properties, protein affinity and metabolic regulation [4–6]. Consequently, chitosan is widely used in wide-ranging biomedical applications, including tissue engineering, drug delivery and medical dressings [7–10]. Chitosan is a linear polysaccharide obtained by deacetylation of chitin [5]. The degree of deacetylation (DDA) determines the proportion of free amine groups residing in its chemical structure [5,11]. The physical properties of chitosan, including its solubility and biodegradability, are markedly affected by DDA [12,13]. Particular biological responses were observed in cells when chitosan polymers with different DDA were applied [14]. Free amine groups in the chemical structure of chitosan polymer are believed to be responsible for associated biochemical effects [15].

To engineer tissue for regeneration, optimizing the properties of an applied biomaterial for tissue-specific requirement is imperative. Our previous studies demonstrated that chitosan contributes to the generation of the branching structure of the salivary gland [3,16–19]. By providing an interactive environment for progenitor salivary tissues, chitosan efficiently promotes the formation of branches. Because DDA markedly alters the biochemical properties of chitosan, DDA is an important factor and must be considered when using chitosan. Until now, the morphogenetic effect of chitosan DDA remains elusive [3,16–18]. Furthermore, the optimal DDA of chitosan for guiding the formation of salivary branches has not yet been determined. To optimize the use of chitosan for tissue regeneration, one must address the impact of DDA on the morphogenetic ability of chitosan to assist in tissue formation. This study aims to investigate the morphogenetic effect of the salivary gland by controlling chitosan DDA. The results provide important information for optimizing chitosan properties for the structural formation of salivary glands.

2. Materials and methods

2.1. Organ culture of salivary gland

Tissue explants of the submandibular gland (SMG) were harvested from E13 ICR mice. Animal protocols were approved by the National Taiwan University Animal Care and Use Committee. The organ culture system and SMG medium were prepared as described previously [3,16–18,20]. Briefly, samples of chitin and chitosan treated for having specific DDA were prepared. They were dissolved in 1 M acetic acid. To prepare the chitin or chitosan-containing medium, these samples were mixed with a serum-free medium and neutralized with sodium hydroxide for a final concentration of 0.3 mg ml^{-1}. A control medium was prepared in the same manner as the chitosan-containing medium without adding chitosan or chitin samples. It was used as the control in the following assays. SMG explants were placed onto the Nuclepore filter (Whatman) suspended in an air–liquid interface on the cultured medium. The serum-free medium is composed of Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (Gibco) medium supplemented with 150 mg ml^{-1} vitamin C, 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin. The explants were cultured at 37 °C in a humidified chamber with 5% CO_2 and 95% air atmosphere. In all assays, SMG explants cultured without chitin or chitosan were controls. The cultured SMG explant was photographed and measured at indicated time-points. Quantitative comparison of the number of SMG branches was based on fold change, which is defined as the ratio change in the number of branches between experimental and control groups [18]. Each experiment was repeated at least three times.

2.2. N-deacetylation of chitin

The procedure of *N*-deacetylation of chitin/chitosan to produce materials with different DDA followed the process reported previously [21]. Chitin obtained from a commercial source (Sigma–Aldrich, St Louis, MO) was used for deacetylation. Different experimental designs for deacetylation, including varying alkaline concentration (40–60%), temperature (40–80 °C) and reaction time (60–120 min), were used to generate chitosan samples with different DDA. Since the chitin/alkaline solution ratio was not a major determinant [21], it was set at 1:10 in all experiments. Fig. 1 represents preparation details for each sample. During the reactions, samples were kept in a silicone oil bath with the indicated temperature. Chitin samples were harvested after a predefined reaction time.

2.3. N-acetylation of chitosan

N-acetylation of chitosan was performed using acetic anhydride as the reactive reagent [22]. Chitosan (448869, DDA 75–85%, Sigma-Aldrich, St Louis, MO) was first resolved in 10 ml 0.5% acetic acid solution, and was then mixed with 8 ml 1,2-propanediol. The acetylating reagent was prepared in a mixture of 2 ml 1,2-propanediol and acetic anhydride. Both solutions were then mixed together slowly and stirred for 24 h [23]. The DDA of a chitosan sample was changed by adding different volumes of acetic anhydride. After reactions, ammonia was added to precipitate chitosan samples. All chitosan samples were washed thoroughly with deionized water and lyophilized.

2.4. Determination of degree of deacetylation and molecular weight of chitosan samples

The DDA of chitosan samples obtained under various conditions was determined by ^{1}H NMR spectroscopy. Spectra were recorded on a Bruker DPX spectrometer (400 MHz) at room temperature. The DDA of each chitosan sample was determined based on the ratio of area between methyl protons of *N*-acetylglucosamine units to that of all H2–H6 protons of both glucosamine and *N*-acetylglucosamine units. The DDA of each sample was calculated using the formula described previously [23,24]. The value of DDA of each sample was compared to that of the original chitosan substrate (Chito100) and is represented as relative DDA. Therefore, five chitosan samples with different DDA were used in the following assays and named Chito100, Chito63, Chito49, Chito15 and Chito7, respectively.

The molecular weight of the chitosan samples was measured using a viscometric method [25]. All samples were prepared in 0.2 M acetic acid and 0.1 M sodium acetate aqueous solutions. The relative viscosity of chitosan samples was determined using an Ubbelohde capillary viscometer at 30 ± 0.5 °C. Intrinsic viscosity is defined as reduced viscosity extrapolated to a chitosan concentration of zero. The viscosity and average molecular weight were calculated using the Mark–Houwink equation [25]. All measurements were repeated at least five times and averages are reported.

2.5. Semi-quantitative reverse-transcription polymerase chain reaction and quantitative polymerase chain reaction

The RNA was extracted from cultured SMG explants using an RNase kit (Qiagen, Valencia, CA), and was reverse transcribed with Superscript III (Invitrogen, Carlsbad, CA). Polymerase chain reaction (PCR) was performed using specific primers for mouse genes encoding type I collagen (Col I, forward, 5′- 5′-GAG-