RESEARCH REPORTS
Clinical

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J Dent Res 85(10):933-936, 2006

Salivary LL-37 Secretion in Individuals with Down Syndrome is Normal

ABSTRACT

Antimicrobial peptides play an important role in the innate immune response. Deficiency in salivary LL-37 antimicrobial peptide has been implicated in periodontitis in patients with morbus Kostman syndrome. Down syndrome is associated with periodontitis, diminished salivary flow, and salivary immunoglobulin deficiency. In the present study, levels of LL-37 and its hCAP18 precursor were measured in saliva samples from young individuals with Down syndrome and compared with levels in those from age-matched healthy controls. LL-37 and human cathelicidin antimicrobial protein (hCAP18) were detected in whole but not in parotid saliva. hCAP18 was more abundant than LL-37. The concentrations of salivary hCAP18 and LL-37 were found to be higher in individuals with Down syndrome than in healthy controls, but their secretion rates were similar. We concluded that, while the adaptive immunity of individuals with Down syndrome is impaired at the oral mucosa, the secretion rate of the LL-37 component of the innate immune system is normal.

KEY WORDS: Down syndrome, saliva, LL-37, hCAP18, innate immunity, antimicrobial peptides.

Received July 7, 2005; Last revision April 27, 2006; Accepted May 16, 2006

INTRODUCTION

Down syndrome is associated with periodontal disease, which develops at an early age and is rapid and extensive (Saxén and Aula, 1982; Shaw and Saxby, 1986; Cichon *et al.*, 1998), while a contrastingly low incidence of caries lesions is characteristically observed (Cutress, 1971; Orner, 1976).

Individuals with Down syndrome suffer from multiple impairments in their systemic immune system, which render them susceptible to infectious disease and malignancies (Desai, 1997). Previously, we have reported severe impairment of acquired oral mucosal immunity in individuals with Down syndrome (Chaushu *et al.*, 2002a), with immunoglobulins IgA and IgG secretion rates in parotid saliva being only 13% and 25%, respectively, of those measured in healthy controls (Chaushu *et al.*, 2002a). An increased vulnerability to recurrent upper respiratory infections was attributed to this reduction in salivary IgA secretion (Chaushu *et al.*, 2002b) and has been suggested as a factor in the higher incidence of periodontal disease in individuals with Down syndrome.

Innate immunity is significant in the remarkable ability of the oral cavity to tolerate injury and recover rapidly from extensive wounds, such as tooth extraction. Migratory cells (such as macrophages and neutrophils), proteins (such as lysozyme), lectins, and complement each contributes to oral innate immunity (Zasloff, 2002b). Antimicrobial peptides are considered to play a major role in the oral cavity's first line of defense (Zasloff, 2002a). These molecules have a direct bactericidal activity and can indirectly stimulate additional immune defense through chemotactic activity or by induction of cytokine release.

Morbus Kostmann syndrome is a genetic disorder, characterized by severe congenital neutropenia, and is highly associated with periodontitis (Putsep *et al.*, 2002). Thus, in this respect, morbus Kostmann syndrome is similar to Down syndrome. Despite treatment with recombinant granulocyte colony-stimulating factor (G-CSF), which restores neutrophil levels, morbus Kostmann patients suffer from severe periodontitis (Defraia and Marinelli, 2001; Putsep *et al.*, 2002). Examination of neutrophils collected from G-CSF-treated morbus Kostmann patients revealed that their mean defensin content was approximately 30% of that of healthy controls. The examined neutrophils were completely defective in LL-37 production, which is undetectable in the patients' saliva. No other dysfunction could be found in neutrophils of G-CSF-treated morbus Kostmann patients. Bone marrow transplantation in a morbus Kostmann patient restored salivary LL-37 secretion and prevented periodontitis. Hence, it has been suggested that periodontitis in morbus Kostmann patients is associated with LL-37 deficiency (Putsep *et al.*, 2002).

Given the similarity between Down syndrome and morbus Kostmann with respect to periodontal disease, we hypothesized that salivary LL-37 deficiency prevails in individuals with Down syndrome as well.

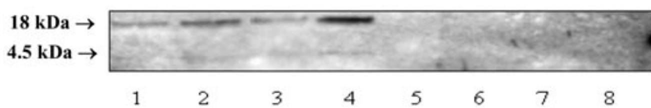

Figure 1. Western blot analysis of hCAP18 (18 kDa) and LL-37 (4.5 kDa) in whole (lanes 1-4) *vs.* parotid (lanes 5-8) saliva. Each lane contains respective saliva samples collected from different (N = 4) control donors.

MATERIALS & METHODS

Patients

The experimental study group included 26 patients with Down syndrome (mean age, 23.7 ± 4 yrs, 13 females and 17 males). Each patient had a typical trisomy of chromosome 21. All patients attended the Elwyn Center for the Disabled (Jerusalem), either as full-time residents or as day residents living in hostel-like apartments. Fifteen of the 26 Down syndrome patients suffered from periodontal disease (examined clinically and radiographically); however, no significant differences were found between these two subgroups in all the parameters evaluated (data not shown). The control group consisted of 16 age-matched healthy volunteers (mean age, 22.3 ± 6 yrs), students and employees of the Faculty of Dental Medicine, in the Hebrew University of Jerusalem. None of the control patients suffered from periodontal disease (examined as above). All participants provided informed consent (provided, in the case of subjects with DS, by a parent or guardian) to a protocol that was reviewed and approved by the institutional ethics committee.

Saliva Collection

The saliva samples were collected between the hours of 9:00 a.m. and 12:00 noon. Unstimulated whole saliva samples were obtained with the aid of a trachea suction set (Unomedical, Kongevejen, Denmark) used in the collection of sputum for microbial culture. The total amount collected over a five-minute period was registered, and the salivary flow rates (mL/min) were calculated. Parotid saliva was stimulated by 100 μL of 2% citric acid applied to the tongue every 15 sec over a period of 10 to 20 min, and was collected with a parotid salivary gland Lashley cannula. In this manner, saliva flow rate was measured from the initiation of the gustatory stimulation, and the parotid salivary flow rates (mL/min) were recorded. Saliva samples were immediately placed in ice, cleared by centrifugation at 12,000 rpm for 2 min (room temperature), and stored at -70°C until assayed.

LL-37 Peptide

LL37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) was synthesized by the solid phase method, on a fully automated, programmable peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA, USA). Peptide integrity and purity (higher than 95%) were determined by analytical high-performance liquid chromatography (HPLC) and mass spectrometry.

Antisera and Western-blot Analysis

Rabbit antibodies were generated against the synthetic LL-37 peptide (Harlan Biotech, Rehovot, Israel), according to a protocol described previously (Bachrach *et al.*, 1994) after approval by the Animal Care and Use Committee of the Hebrew University of Jerusalem. Antiserum specificity was confirmed by Western immunodetection analysis of human saliva and of human serum. In both cases, the antibodies identified 2 discrete bands with the expected molecular weights corresponding to hCAP18 and LL-37. Identical bands were also detected with a monoclonal antibody against LL-37 (Hycult Biotechnology, Uden, The Netherlands). Western blot immunodetection was carried out according to procedures previously described (Towbin *et al.*, 1979; Rozen *et al.*, 2004). Saliva sample (22 μL) proteins were separated on a 15% SDS-acrylamide gel and transferred to polyvinylidenedifluoride (PVDF) membranes. Blots were blocked with Tween-Tris-buffered saline (TTBS) (50 mM Tris-HCl [pH 7.4], 200 mM NaCl and 0.04% Tween 20) containing 5% non-fat dry milk. Rabbit anti-LL-37 serum was used at a 1:250 dilution, and peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used at a 1:10,000 dilution. Following incubation with the antibodies, blots were washed 3x with TTBS, and exposed to SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA). Bands were visualized by means of a CL-BIS reader (DNR, Kiryat Anavim, Israel) chemiluminescence detector and assessed by the "ImageJ" densitometry software (NIH, Bethesda, MD, USA).

LL37 and hCAP18 concentrations were calculated based on a calibration curve of synthetic LL-37 peptide at known concentrations. We calculated LL37 and hCAP18 salivary secretion rates in μg/min by multiplying peptide concentrations by salivary flow rate.

Statistical Analysis

The minimal sample size of the studied groups was estimated according to the formula $n = (t^2 \times SD^2)/L^2$, where n = number of patients, $t = 1.96$ for a confidence level of 95%, SD = standard deviation, and L = relative error to the mean, which is the product of sample mean χ and precision p expressed as a fraction (Cochran, 1977). The minimal estimated sample size, for achievement of a precision of ± 50% of the mean with 95% certainty, was 13 for the

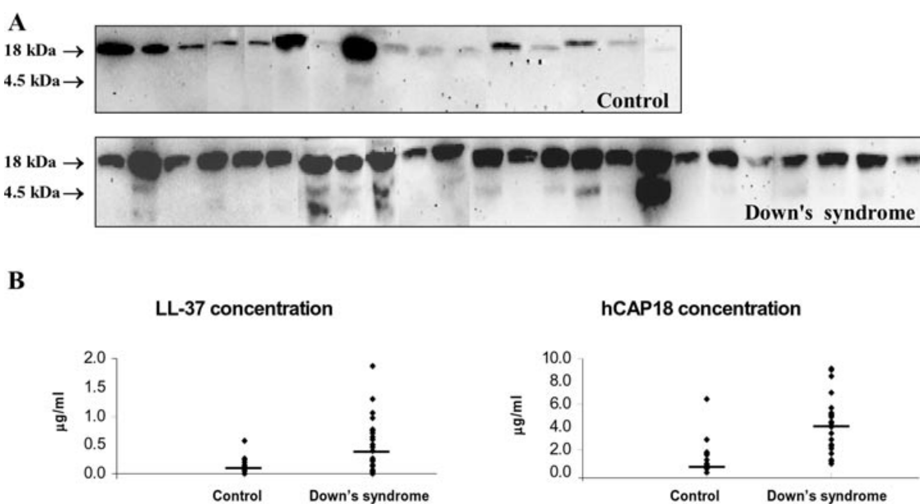

Figure 2. Western blot analysis **(A)** and calculated concentrations **(B)** of hCAP18 (18 kDa) and LL-37 (4.5 kDa) in whole-saliva samples collected from control (N = 16) and from Down syndrome (N = 24) donors.

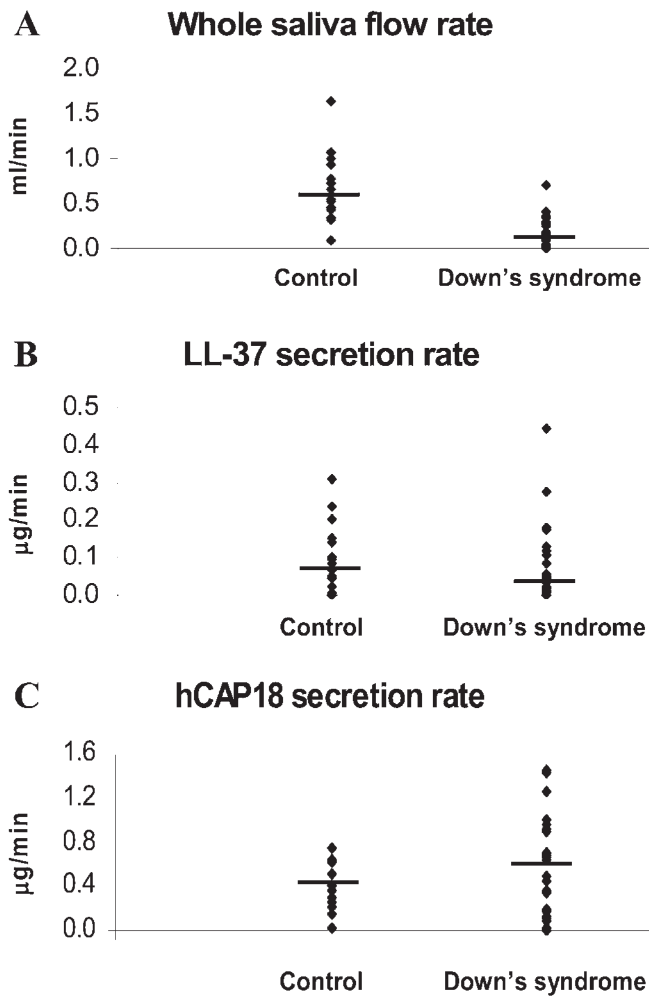

Figure 3. Flow rate of whole saliva **(A)** and secretion rate of LL-37 **(B)** and hCAP18 **(C)** in whole saliva of individuals with Down syndrome (N = 26) *vs.* healthy controls (N = 16).

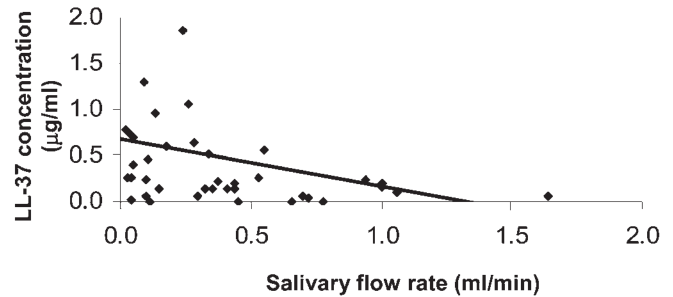

Figure 4. Effect of salivary flow rate on LL-37 concentrations in whole saliva (N = 40).

secretion rates and 14 for concentrations. Differences between groups were evaluated, analyzed, and compared by the non-parametric Wilcoxon rank test. Correlation between LL-37 concentration and whole-saliva flow rate was measured by the non-parametric Kendall test. All p values given are based on two-tailed tests, and $p < 0.05$ was the criterion of significance. Data analysis was performed with the SAS statistical analysis software.

RESULTS

LL-37 and its precursor, hCAP18, could be detected by Western immunodetection in whole saliva (Fig. 1). The median concentrations of hCAP18 were 4.3- and 12-fold higher than those of LL-37 in the control and Down syndrome groups, respectively ($P < 0.001$) (Fig. 2).

The median concentrations of LL-37 and of hCAP18 in clarified whole saliva collected from young individuals with Down syndrome (0.33 µg/mL and 4.26 µg/mL, respectively) were significantly higher ($p = 0.02$ and $p < 0.01$, respectively) than those measured in clarified whole saliva collected from an age-matched group of healthy controls (0.14 µg/mL and 0.60 µg/mL) (Fig. 2).

The median whole-saliva flow rate measured in individuals with Down syndrome was significantly lower than that measured in healthy controls (0.12 mL/min compared with 0.60 mL/min, $p < 0.01$, Fig. 3). However, median LL-37 and hCAP18 secretion rates in whole saliva (calculated in µg/min by multiplying peptide concentration by salivary flow rate) in young individuals with Down syndrome (0.04 µg/min and 0.64 µg/min, respectively) were not significantly different ($p = 0.30$ and $p = 0.83$, respectively) from those calculated in age-matched healthy controls (0.07 µg/min and 0.45 µg/min, respectively, Fig. 3).

LL-37 and hCAP18 were not detected in stimulated parotid saliva. The median parotid saliva flow rate was significantly lower in individuals with Down syndrome than in healthy controls (0.10 mL/min compared with 0.21 mL/min, respectively, $p < 0.01$).

DISCUSSION

Down syndrome individuals have an increased prevalence of periodontal disease compared with normal and other mentally compromised patients of similar age distribution (Cutress, 1971; Orner, 1976; Saxén *et al.*, 1977; Barnett *et al.*, 1986; Reuland-Bosma and van Dijk, 1986). We have reported previously that the oral mucosal adaptive immunity of individuals with Down syndrome is defective (Chaushu *et al.*, 2002a), thus exposing them to recurrent respiratory infections (Chaushu *et al.*, 2002b). In this study, we found that although young individuals with Down syndrome have a diminished salivary flow rate compared with that in an age-matched control group, their LL-37 secretion rate in whole saliva remains normal. Since we clarified whole saliva by centrifugation to facilitate Western blot immunodetection, it is possible that some LL-37 and hCAP18 were removed with the sediment containing the mucin precipitate. Plotting the salivary LL-37 concentrations *vs.* whole saliva flow rate revealed an inverse correlation between salivary flow rate and LL-37 concentration, in both Down syndrome and healthy controls ($R = -0.3$, $P = 0.008$) (Fig. 4). Thus, a higher LL-37 concentration in whole saliva compensated for lower salivary flow rate.

LL-37 is expressed in neutrophils (Cowland *et al.*, 1995), epithelial cells, acinar cells of the submandibular gland, the palatine minor glands, lingual epithelium, and in the palatal mucosa (Murakami *et al.*, 2002). Here, we found that the secretion rate of salivary LL-37 was not influenced by the rate of salivary flow. This is in contrast to salivary IgA, which subsided in individuals with Down syndrome, in the same way as did salivary flow rate (Chaushu *et al.*, 2002a).

LL-37 has been reported to be expressed in the glandular

epithelium of the human parotid glands (Woo *et al.*, 2003). In our study, LL-37 was not detected in parotid saliva. Therefore, we have reason to believe that LL-37 is scarcely secreted into parotid saliva, and that it is expressed in the parotid gland primarily to protect the glandular cells from retrograde infections, as suggested previously (Woo *et al.*, 2003).

If LL-37 is secreted into whole saliva by oral epithelial cells, and primarily by neutrophils present in the oral cavity, a lower salivary flow rate would be expected to result in an increase in salivary LL-37 concentration. This conclusion supports the finding that neutrophil-defective patients with morbus Kostmann are deficient in salivary LL-37 (Putsep *et al.*, 2002).

Parallel to other body fluids (Frohm *et al.*, 1996; Sorensen *et al.*, 2001; Andersson *et al.*, 2002; Ong *et al.*, 2002), concentration of salivary LL-37 was lower than that of its hCAP18 precursor.

Overall, our study demonstrated that LL-37 salivary output in individuals with Down syndrome (LL-37 secretion rates) was normal. Thus, we conclude that normal salivary LL-37 levels are insufficient to protect against periodontitis when accompanied with deficiencies in the oral mucosal acquired immunity (IgA) (Chaushu *et al.*, 2002a), and innate immunity (neutrophils).

Caries-associated Gram-positive oral bacteria were found to be more susceptible to cationic antimicrobial peptides (including LL-37) compared with the primarily Gram-negative oral pathogens associated with periodontitis (Ouhara *et al.*, 2005). It is tempting to speculate that, similar to LL-37, other oral cationic antimicrobial peptides might be found in normal amounts in the saliva of individuals with Down syndrome. Their presence at adequate levels may provide an explanation as to why periodontitis is prevalent in individuals with Down syndrome, while caries is conspicuously absent.

ACKNOWLEDGMENTS

This investigation was supported by the Izadore I. Cabakoff Research Endowment Fund of Hebrew University-Hadassah School of Dental Medicine. We thank Prof. A. Becker for his helpful discussion; Dr. Ilan Feldberg, Chief Dental Consultant, Division for Mental Retardation, Ministry of Social Affairs; and the staff in the Dental Clinic of Elwyn Center for the Disabled, especially Mrs. Ofra Saadon and Dr. Moshe Einhorn, for their cooperation in this study.

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