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Investigation of serum and saliva dermcidin levels in patients with recurrent aphthous stomatitis and dermcidin analysis in salivary gland

Rekürren aftöz stomatitli hastalarda serum ve tükürükte dermcidin düzeylerinin araştırılması ve tükürük bezinde dermcidin analizi

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Abstract

Objective Recurrent aphthous stomatitis (RAS) is a common self-limiting oral mucosa disease. In this study, it was aimed to determine the dermcidin level in the serum and saliva of patients with RAS, the presence of dermcidin in the salivary gland and its role in the pathogenesis of RAS.

Methods Thirty-one patients presenting with RAS and 30 control subjects participated in this study. Dermcidin levels in serum and saliva of patients and control group were studied in accordance with the working procedures specified in the catalogs of the human dermcidin ELISA kit. The presence of dermcidin in salivary glands was assessed by immunohistochemical analysis.

Results A statistically significant difference was found when the mean salivary dermcidin levels (105.80 ± 80.14 ng/mL) of the RAS patients were compared with the mean salivary dermcidin levels (456.13 ± 354.59 ng/mL) of the control group (P=0.000). There was no statistically significant difference between the mean serum dermcidin levels (316.41 ± 784.55 ng/mL) of the RAS patient and those of (130.65 ± 179.75 ng/mL) the control group. Dermcidin immunoreactivity was observed in the parotid gland, submandibular gland and interlobular striated ducts.

Conclusion The findings in this study showed that striated cells in salivary gland synthesized dermcidin. Low levels of dermcidin with antimicrobial properties in saliva were considered as a predisposing factor for RAS.

Key words: *dermcidin, recurrent aphthous stomatitis, salivary gland*

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Öz

Amaç Rekürren aftöz stomatit (RAS) dudak mukozası, yanak ve dilde, tekrarlayıcı, küçük, ağrılı, eritemli halesi bulunan, nekrotik ülserlerle karakterize, kendi kendini sınırlayabilen, sık görülen bir oral mukoza hastalığıdır. Bu çalışmada RAS'lı hastaların serum ve tükürüğünde dermcidin düzeyinin, tükürük bezinde dermcidin varlığının ve RAS patogenezindeki rolünün belirlenmesi amaçlandı.

Yöntem Bu çalışma 31 hasta ve 30 sağlıklı gönüllü ile yapıldı. Hasta ve kontrol grubunun serum ve tükürüğündeki dermcidin düzeyleri, insan dermcidin ELISA kitinin kataloglarında belirtilen çalışma prosedürlerine göre çalışıldı. Tükürük bezlerinde dermcidin varlığı immünohistokimyasal analiz ile değerlendirildi.

Bulgular RAS'lı hasta grubunun ortalama tükürük dermcidin düzeyleri (105.80 ± 80.14), kontrol grubunun ortalama tükürük dermcidin düzeyleri (456.13 ± 354.59) ile kıyaslandığında istatistiksel anlamlı farklılık tespit edildi (P=0.000). RAS'lı hasta grubunda ortalama serum dermcidin düzeyleri (316.41 ± 784.55), kontrol grubunun ortalama serum dermcidin düzeyleri (130.65 ± 179.75) ile kıyaslandığında istatistiksel anlamlı farklılık tespit edilemedi. İmmünohistokimyasal boyamada parotis ve submandibular bezlerde, interlobular kanallarda dermcidin immünreaktivitesi gözlendi.

Sonuç Sonuç olarak bu çalışmada tükürük bezinin striated hücrelerinin dermcidin sentezlediği ortaya kondu. Tükürükte antimikrobial özellikli dermcidin azlığının RAS için predispozan bir faktör olduğu düşünüldü.

Anahtar kelimeler: *dermcidin, rekürren aftöz stomatit, tükürük bezi*

Introduction

Recurrent aphthous stomatitis (RAS) is a common self-limiting oral mucosa disease characterized by necrotic ulcers. Many agents, such as genetic factors, food allergies, local trauma, vitamin and element deficiencies, endocrine factors, stress, smoking cessation, chemical substances, viral and bacterial infections have been accused of its etiology.[1,2] The pathogenesis of RAS has been explained by the activation of proinflammatory cytokines leading to the damage of the oral mucosa under the influence of triggering factors on the basis of genetic susceptibility.[2] It is known that RAS is associated with microbial agents such as *Streptococcus sanguis* and inflammatory markers such as TNF-α.[3]

Dermcidin was discovered by Schitteck et al. in 2001. The peptide is released from the sweat glands as a precursor protein with a weight of 9.3 kD, cleaved by proteolytic enzymes and converted into small peptides with antimicrobial properties.[4] Some studies have indicated that that the peptides derived from the dermcidin have antibacterial activity against *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis*[4], *Candida albicans*[5], *Staphylococcus epidermidis*[6], *Pseudomonas putida*, rifampicin- and isoniazid-resistant *Mycobacterium tuberculosis*[7] *and P. acnes.*[8] Also, it was detected that the concentration of dermcidin in the skin of patients with tinea pedis was low, which suggests that dermcidin may be mycostatic activity and may prevent fungal colonization.[9]

This study was planned because of the antimicrobial properties of the dermcidin molecule, and the presence of microbial agents in the etiology of RAS. It is notable that there is no research in the literature that investigating the serum and salivary dermcidin levels of RAS patients. To contribute to the relevant literature, in the present research, we aim to determine the level of dermcidin in the serum and saliva of patients, the presence of dermcidin in the salivary gland, and its role in the pathogenesis of RAS.

Methods

Thirty-one patients presenting with RAS and 30 control subjects participated in this study. This study was approved by the ethics committee (30.09.2014, no:02) and conducted at a dermatology outpatient clinic. The patient group consisted of participants older than 18

years old who had clinically recurrent aphthous lesions in the oral mucosa and no other underlying disease. The control group consisted of individuals aged 18 years old or older who applied to the hospital for the annual check-up. Pregnancy, diabetes, hypertension, hyperthyroidism and hypothyroidism, malignancy, alcohol-drug abuse and any systemic drug treatment were considered as an exclusion criteria. Patients and control group were informed about the study and then they gave informed consent.

To obtain saliva, participants were allowed to spit for 1-2 minutes into the sterile urine culture containers after 5 minutes they thoroughly gargled their mouths. A stimulation test was not performed to obtain saliva. 1-2 mL of saliva was taken into urine culture containers that include the same amount of aprotinin. The samples in the eppendorf tubes were stored at -80°C. 5 mL fasting blood sample from each participant was taken in the morning. Since dermcidin is a hormone in peptide structure, before receiving blood from the participants, 500 mL of kallikrein unite aprotinin for 1 mL was added to the tubes to prevent its disintegration by proteases. After receiving, blood samples were centrifuged, then they were transferred to the eppendorf tubes and stored in the deep freezer (-80°C) until the analysis.

Immunohistochemical analysis of dermcidin in serum and saliva

Dermcidin levels in serum and saliva of patients and control group were studied in accordance with the working procedures specified in the catalogs of the human dermcidin ELISA kit Sunred Bioscience (Catalog ID: 201-12-5460 Shanghai, CHINA). Intra-Assay CV value of the reagent was <10%, while Inter-Assay CV value was <12%. Plate washes were performed with an automatic washer Bio-Tek ELX50 instrument (BioTek Instruments, USA) and absorbance measurements were performed by ChroMate, Microplate Reader P4300 instrument (Awareness Technology Instruments, USA). Test results were reported as ng/mL. The reference range was considered as 1 ng/mL-300 ng/mL while sensitivity value was 0.903 ng/mL.

Immunohistochemical analysis of dermcidin in the salivary gland was performed with salivary gland tissue without any pathology (n:10), which was previously excised for any reason and sent to pathological examination. Immunohistochemical staining of the tissues was performed using the method of Hsu et al.[10] The amount of dermcidin in the tissue was measured by the ELISA method[11] after the saliva gland was grinded in the phosphate buffer. Anti-DCD/Dermcidin antibody (aa96-110) produced by LSBio (Life Span BioSciences, Inc.) (Catalog ID/Lot ID: LS-C128574/32734) was used for the measurement.

Sections with 5-6 mm in thickness taken from paraffin blocks were transferred into the slides with polylysine. The deparaffinized tissues were passed through graded alcohol series and boiled in the microwave (750W) for 7+5 minutes at pH:6 in citrate buffer solution for antigen retrieval. The tissues that were left to cool in the room temperature for about 20 minutes after boiling were incubated with hydrogen peroxide block solution for 5 minutes (Hydrogen Peroxide Block, TA-125-HP, Lab Vision Corporation, USA) to prevent endogenous peroxidase activity after washing for 3x5 minutes with PBS (Phosphate Buffered Saline, P4417, Sigma-Aldrich, USA). After Ultra V Block solution (TA-125-UB, Lab Vision Corporation, USA) was applied for 5 minutes to the tissues washed with PBS for 3x5 minutes to prevent floor paint, 1/200 of diluted primary antibody (Anti-DCD/Dermcidin Antibody, aa96-110, Life Span BioSciences, Inc., Seattle, USA) was incubated for 60 min in a humid environment at room temperature. The tissues were incubated at room temperature for 30 minutes in a humidified environment with a secondary antibody (biotinylated Goat Anti-Polyvalent (anti-mouse/rabbit IgG 80°C), TP-125-BN, Lab Vision Corporation, USA) after washing with PBS for 3x5 minutes and application of the primer antibody. The tissues were washed with PBS for 3x5 minutes after the application of secondary antibody, and then, incubated with PBS (Streptavidin Peroxidase, TS-125-HR, Lab Vision Corporation, USA) for 30 minutes at room temperature in humidified atmosphere and finally transferred to the PBS. After

the solution of 3-amino-9-ethylcarbazole (AEC) Substrate+AEC Chromogen (AEC Substrate, TA-015 and HAS, AEC Chromogen, TA-002-HAC, Lab Vision Corporation, USA) were added to the tissues. The vision signal was taken on the light microscope, the tissues were simultaneously washed with PBS. Mayer's hematoxylin-counterstained tissues were covered with the appropriate closure solution (Large Volume Vision Mount, TA-125-UG, Lab Vision Corporation, USA) after the applications of PBS and distilled water. Preparations were photographed by examining on a Leica DM500 microscope (Leica DFC29580°C).

Statistical analysis

SPSS version 22.0 was used for statistical analysis. The values obtained in the study were given as mean ± SD. Student t-test and Mann-Whitney-U test were applied for inter-group comparisons. $P<0.05$ were considered statistically significant.

Results

A total of 61 participants consisting of 28 (45.9%) female and 33 (54.1%) male were included in the present study. A total of 31 patients consisting of 15 (48.4%) female and 16 (51.6%) male were enrolled in the patient group, while there were 30 volunteers as 13 (43.3%) female and 17 (56.7%) male in the control group. Patients were in the age range between 21-55 years, with a mean age of 34.22 ± 9.00 and the age range was between 18-48 years and mean age was 34.80 ± 8.00 in the control group (Table 1).

Table 1. Demographic and laboratory characteristics of the patient and the control group

Parameters	RAS	Control	*P*
n	31	30	
Gender (M/F)	16/15	17/13	*P*>0.05
Age* (year)	34.22 ± 9.00	34.80 ± 8.00	*P*>0.05
Serum Dermcidin (ng/mL)	316.41 ± 784.55	130.65 ± 179.75	*P*>0.05
Saliva Dermcidin (ng/mL)	105.80 ± 80.14	456.13 ± 354.59	*P*=0.000

*(Mean ± SD)
RAS, Recurrent aphthous stomatitis

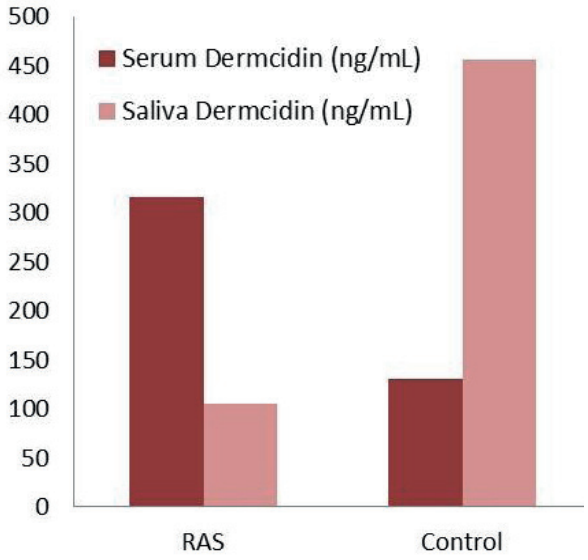

Fig. 1. Serum and saliva dermcidin levels of the patients and control groups

A statistically significant difference was found when the mean salivary dermcidin levels (105.80 ± 80.14 ng/mL) of the RAS patients were compared with the mean salivary dermcidin levels (456.13 ± 354.59 ng/mL) of the control group ($P=0.000$). There was no statistically significant difference between the mean serum dermcidin levels (316.41 ± 784.55 ng/mL) of the RAS patient and those of (130.65 ± 179.75 ng/mL) the control group. (Table 1) (Fig. 1).

Although serum dermcidin levels were higher in women with RAS (489.59 ± 1068.63 ng/mL) than men with RAS (154.05 ± 327.23 ng/mL), the difference was not statistically significant. Similarly, although the salivary dermcidin levels in patients were higher

in female patients (120.77 ± 112.42 ng/mL) than male patients (91.77 ± 24.81 ng/mL), the difference was not statistically significant. Also, for the control group serum and saliva dermcidin levels were higher in females than males but these differences were not statistically significant. In addition, a significant correlation was not found out between serum and salivary dermcidin levels.

Parotid (Fig. 2a) and submandibular glands (Fig. 2b) were used to investigate dermcidin immunoreactivity. Dermcidin immunoreactivity was observed in the parotid gland (Fig. 2c) and submandibular gland (Fig. 2d) and interlobular ducts.

Discussion

In this study, to our knowledge, dermcidin concentrations in saliva of patients were determined for the first time. Also, in this study, salivary glands contribute to dermcidin levels are investigated. In addition, an immunohistochemical scanning of dermcidin was performed on the salivary glands to detect the source of the dermcidin in the saliva. Our findings showed that there was immunoreactivity of dermcidin in striated portions of submandibular and parotid salivary glands.

Saliva has many functions, including moisturizing,

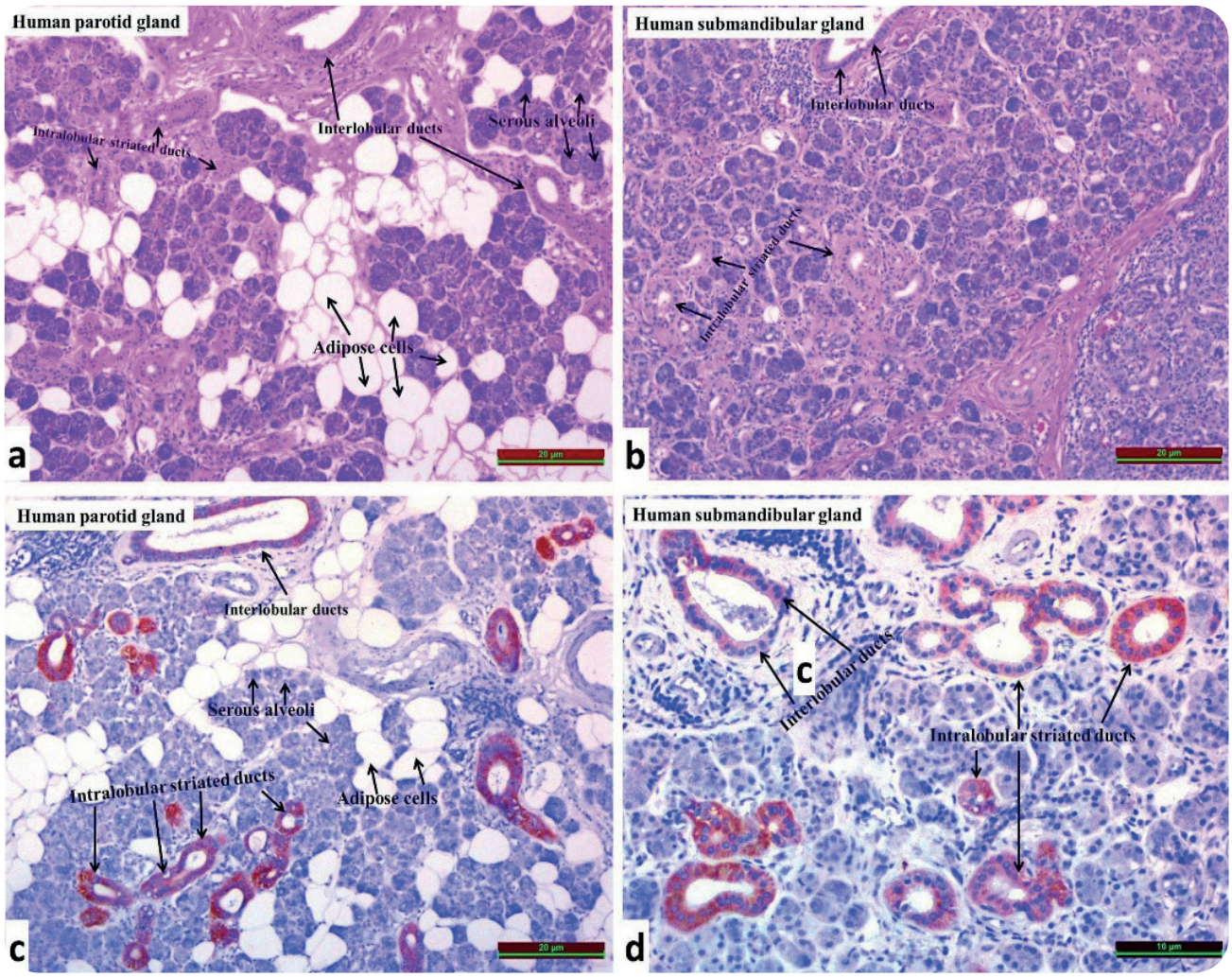

Fig. 2. Parotid **(2a.)** and submandibular **(2b.)** glands, dermcidin immunoreactivity in parotid **(2c.)**, and submandibular glands **(2d.)** and interlobular ducts

lubrication and cleaning of the oral cavity, helping digestion and talking, contributing to dental health, antimicrobial and immunological properties.[12] Many salivary proteins and antimicrobial peptides contribute for the defense system of saliva.[13] Antimicrobial peptides form a natural antibiotic layer on the surface of the oral mucosa as a strong member of the innate immune response and activate the acquired immunity against pathological conditions.[14] Antimicrobial peptides detected in saliva are α and β-defensins, histatins, LL-37 and cathelicidin.[15] Previous proteomic studies have also reported the presence of dermcidin in the saliva; however, it was not detected which cells of the salivary gland synthesize dermcidin.[16,17] Therefore, to our knowledge, this is the first study that showed which cells of the salivary glands synthesized dermcidin.

In a previous study, peptides deriving from dermcidin could not be detected in body fluids, such as nasal secretion, tears and saliva[18], while the analysis of dermcidin was performed in cervicovaginal fluid[19] and tear[20] in some other studies. It has been thought that dermcidin is not a peptide with a high concentration in body fluids.[21] In this study, it was shown that the mean dermcidin level of 456 ng/mL (0.456 µg/mL) in the saliva of healthy controls.

The antimicrobial action mechanism of dermcidin in saliva is unknown. However, relevant studies reported that the peptides derived from dermcidin exhibit antimicrobial effect without permeabilization to microbial membranes.[5,22] It has been shown that dermcidin may contribute to cutaneous immunity by releasing various cytokines, such as TNF-α, interleukin-8 with activating keratinocytes.Dermcidin was unaffected by salt, pH, and inflammatory media.[22] and was at an excessively stable level.[21]

In this study, salivary dermcidin levels in patients decreased. In the light of this information and results obtained in this research, it was detected that patients were sensitive to microbial agents on the basis of genetic susceptibility and considering the fact that antimicrobial dermcidin was also capable of triggering inflammation. Thus, the findings suggest that low dermcidin levels in the saliva result in a predisposition to infections and contribute to the development of aphthae.

In this study, how the level of antimicrobial dermcidin in saliva changes as well as the relationship between its salivary level, and its serum level were investigated in patients. The dermcidin synthesized from the salivary gland appears to be transferred to both the saliva and the serum. Since salivary dermcidin levels in controls were higher than its serum levels, the findings suggest that the main source of dermcidin in serum was salivary gland and this condition could be contribute to the serum level of dermcidin synthesized in the salivary gland. However, it is notable that that salivary and serum dermcidin levels of the patients were not parallel, and there was not a significant difference between patients and the control group regarding serum dermcidin levels. This finding suggests that salivary dermcidin rather than serum may be a candidate biomarker of the disease in patients.

The findings in this study showed that striated cells in salivary gland synthesized dermcidin. Low levels of dermcidin with antimicrobial properties in saliva were considered as a predisposing factor for RAS. In light of insightful findings, this study sheds light on novel treatment methods.

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Ethics committee approval:

Local Ethics Committee approval was obtained (30.09.2014, no:02)

Informed consents: They were obtained.

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Conception and design, or analysis and interpretation of data: BD, DC, IE, SA, OU, TK, MK, MY

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