Dentistry
ISSN: 2161-1122

Kumagai et al., Dentistry 2019, 9:4

Research Open Access

Protein Carbonylation as the Pathogenesis of Oral Hyperkeratosis: A Pilot Study

Akiko Kumagai[1]* and Koichi Tsunoda[2]

[1]*Division of Forensic Odontology and Disaster Oral Medicine, Iwate Medical University, Iwate, Japan*

[2]*Departments of Dentistry and Oral Surgery, Oshu City National Health Insurance Magokoro Hospital, Japan*

[*]**Corresponding author:** Akiko Kumagai, Division of Forensic Odontology and Disaster Oral Medicine, Iwate Medical University, Shiwa-Gun, Iwate, Japan, Tel: +81 19 651 5111; E-mail: Kumagaia@iwate-med.ac.jp

Received date: May 23, 2019; **Accepted date:** June 10, 2019; **Published date:** June 20, 2019

Abstract

Objective: We examined the etiology of hyperkeratosis of the oral mucosa, focusing on antioxidant actions and oxidative stress related to the accumulation of oxidized and damaged molecules.

Materials and Methods: The subjects were non-smoking females with squamous cell hyperplasia, oral lichen planus, epithelial dysplasia, or squamous cell carcinoma. Proteins were extracted from hyperkeratosis tissue specimens. Carbonylated proteins, serving as oxidative stress markers, were detected by western blotting and identified by nano-liquid chromatography-tandem mass spectrometry. In addition, we performed immunohistochemical staining of oral mucosa tissue sections using an anti-hexanoyl-lysine (HEL) antibody.

Results: Several carbonylated proteins from hyperkeratosis tissue of the oral mucosa were detected by western blot and identified asalpha-actinin-1 isoform a, tumor rejection antigen (gp96) 1, alpha-actinin 4, and neutral alpha-glucosidase AB isoform 3 precursors. On immunohistochemical staining with the anti-HEL antibody, the prickle to basal cell layers in cornified lesions were positive. These results indicate the presence of local oxidative stress-induced changes in hyperkeratosis tissues and suggest a new approach for treating oral mucosal keratotic lesions.

Keywords: Oral hyperkeratosis; Oxidative stress; Aging; Protein carbonylation; Anti-hexanoyl-lysine antibody

Introduction

Oxidative stress, caused by reactive oxygen species, results in various undesirable biological changes. Enhanced protein carbonylation, which is an oxidative stress marker, is the cause of some of these changes in the skin, including discoloration and keratinization with aging [1]. This suggests that keratinization of the oral mucosa is also influenced by protein carbonylation.

To investigate the cause of oral mucosal keratotic lesions, we performed biochemical and molecular biology studies to examine localized factors of the oral mucosa as oxidative stress biomarkers.

Materials and Methods

Nineteen patients, aged in the range 58-83 years old (median age: 73 years), examined at the Department of Oral and Maxillofacial Surgery, Dental Center of University Hospital and the Department of Dentistry and Oral Surgery, National Health Insurance Hospital, between April 2013 and March 2015, were enrolled in the study. All patients provided consent after explanation of the study objective. Subjects with keratotic lesions but without any ulcers or erosion were selected. Subjects enrolled in the study included 2 patients with squamous cell hyperplasia, 12 oral lichen planus patients diagnosed histopathologically or clinically based on the presence of clinical reticular or reticular erosive formation [2], 2 patients with histopathologically diagnosed epithelial dysplasia, 1 patient with squamous cell carcinoma, and 2 patients with no oral mucosal keratotic lesions as controls. When subjects with cigarette smoking and alcohol consumption habits were excluded [3], the remaining subjects were mostly female. Thus, the subjects of this study were limited to non-smoking females.

Samples (200 mg) of oral mucosal lesions were collected for histopathological and biochemical examinations from the subjects under local anesthesia. This study followed the Declaration of Helsinki on medical protocol and ethics. The Regional Ethical Review Board of Medical University School of Dentistry approved the study (approval no. 01187). Samples extracted from the oral mucosa were washed completely free of blood with saline. Proteins were solubilized with an ultrasonic probe and extracted using a Ready PrepTM Protein Extraction Kit (Bio-Rad, Hercules, CA, USA). The protein in the samples were quantitated by the method of Bradford, using Protein Assay (Bio-Rad) and a microplate reader (Model 680XR, Bio-Rad).

Carbonyl components of proteins, serving as oxidative stress markers, were assayed using a Protein Carbonyl Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). The relative absorbance of the samples was measured at 375 nm against the blank control using a microplate reader. The results were compared with the subjects' ages.

Because the carbonyl groups of proteins readily condense with 2,4-dinitrophenylhydrazine (DNPH), protein carbonyls can be assessed by adding DNPH reagent to form phenylhydrazone, followed by detection of the reaction product using an anti-dinitrophenyl antibody [4].

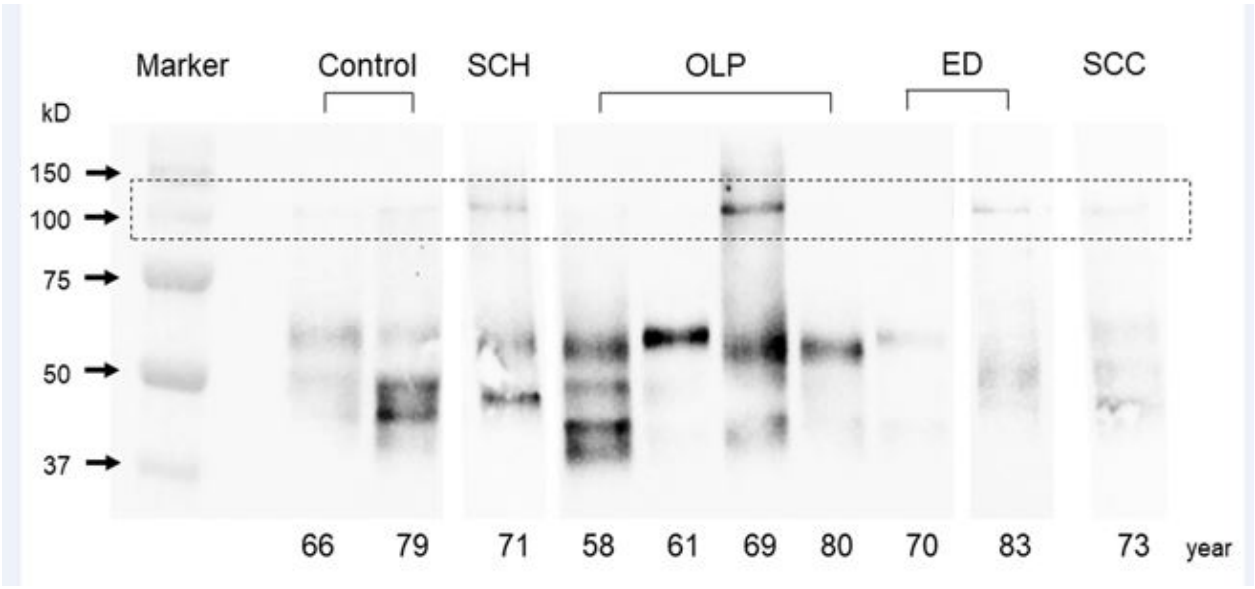

Figure 1: Immunodetection images of carbonyl proteins after SDS-PAGE. SCH=Squamous Cell Hyperplasia; OLP=Oral Lichen Planus; ED=Epithelial Dysplasia; SCC=Squamous Cell Carcinoma. Color development of about 100 kD protein, that was indicated by a square with a dotted line, was unclear in non-keratotic lesions, but a band with color development was tended to observe in 9 of the 17 patients with keratotic lesions (53.0%): 6 of the 12 OLP patients, one of the 2 ED patients, and one each of the SCH and SCC patients.

Extracted proteins were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after derivatizing with DNPH and detected using an OxyBlotTM Protein Oxidation Detection Kit (EMD Millipore, Billerica, MA, USA). SDS-PAGE was performed on two gels (CriterionTM TGXTM Precast Gel, 12%) simultaneously using the CriterionTM cell system (Bio-Rad) with a constant voltage of 200 V for 70 min.

Western blotting for detecting protein carbonyls was performed on one of the two gels after electrophoresis was completed. Proteins on the gel were transferred electrophoretically to an Immun-Blot polyvinylidene difluoride membrane (Bio-Rad) using 15 V, 150 mA, for 30 min. Membranes were incubated with the rabbit anti-dinitrophenyl antibody for 1 h. Those were then washed and the secondary horseradish peroxidase-labeled anti-rabbit IgG antibody was added and incubated for 1 h. After washing, the ELC Prime Western Blotting Detection Reagent (GE Health care, Chicago, IL, USA) was used, and carbonylated proteins were detected using a ChemiDocTMXR Plus system (Bio-Rad).

The second SDS-PAGE gel was stained with 0.4% Coomassie brilliant blue R-250. To identify immunoreacted proteins, bands corresponding to the western blot gel were cut from the stained gel, digested with trypsin, and analyzed by nano-liquid chromatography-tandem mass spectrometry (nano LC-MS/MS, outsourced to Medical and Biological Laboratories, Nagoya, Japan).

Tissue sections were subjected to immunohistochemical staining using an anti-hexanoyl-lysine (HEL) antibody to detect an early step of lipid peroxidation. This technique differs from the ones employing conventional aldehyde lipid peroxide oxidative markers, such as 4-hydroxy-2-nonenal and malondialdehyde, that detect late steps of lipid peroxidation [5]. HEL was localized by observing its expression in positive cells (outsourced to Geno Staff Co., Ltd., Tokyo, Japan). In addition, four or more fields of view were randomly selected from each pathological image for quantification of positive cells. The average percentages of positive cells out of total cells in each group were compared by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test with Microsoft Excel 2016 (Microsoft Corp., Redmond WA, US). $p<0.05$ was considered to indicate statistical significance.

Results

When carbonyls on proteins extracted from the tissues were quantified, no correlation with the age of the patients was observed ($r=0.01$). Upon detection by western blot, protein carbonylation was observed widely in the mid-low protein molecular weight range in both oral mucosal keratotic and non-keratotic lesions (Figure 1). Color development of proteins at about 100 kD was unclear in non-keratotic lesions, but a band with color tended to develop in approximately half of the subjects with keratotic lesions, especially in oral lichen planus lesions. Proteins of the 100 kD band were identified by nano LC-MS/MS. Carbonylation of alpha-actinin-1 isoform a, tumor rejection antigen (gp96) 1, alpha actinin 4, and neutral alpha-glucosidase AB isoform 3 precursor was observed.

Upon staining of oral mucosal keratotic lesions using the anti-HEL antibody, compared with the control, marked zonal color development of the epulis was noted in the basal cell layer, especially in epithelial dysplasia lesions (Figure 2).

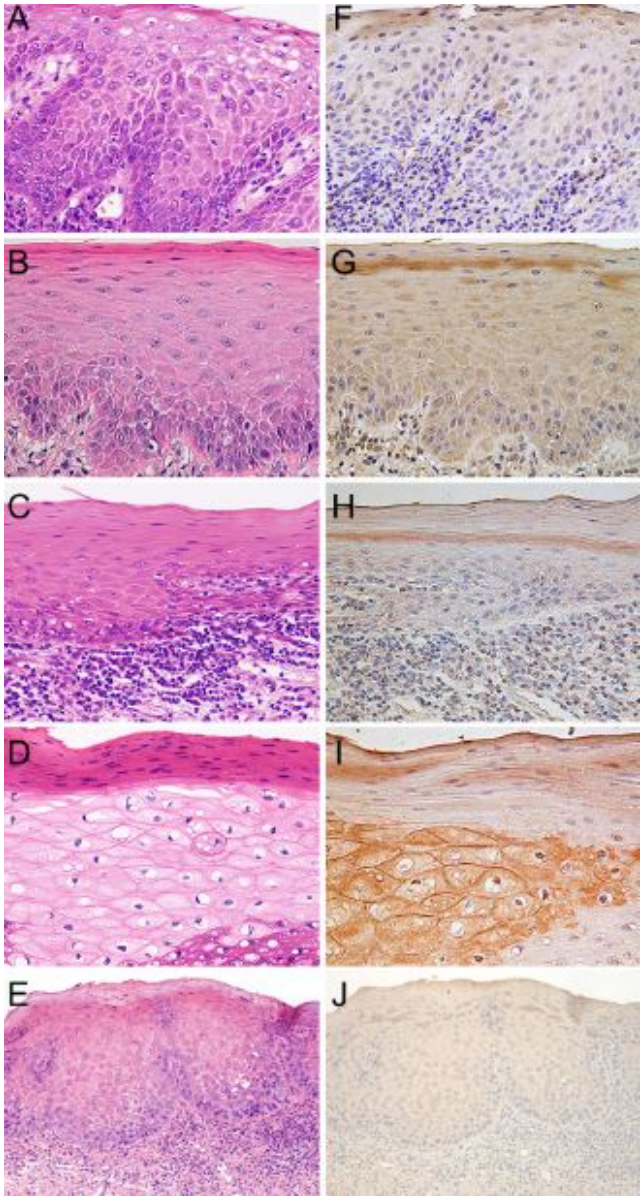

Figure 2: Hematoxylin and eosin staining images (left), Immunohistochemical images by anti-HEL antibody (right). (A) and (F) Epulis as a control. (B) and (G) Squamous cell hyperplasia. (C) and (H) Oral lichen planus. (D) and (I) Epithelial dysplasia. (E) and (J) Squamous cell carcinoma (low power field). Compared with the control, marked zonal color development by reaction with anti-HEL antibody was noted until the basal cell layer, especially in LE lesions.

HEL localization was clear, suggesting that lipids in the oral mucosal keratotic lesions were peroxidized. The percentages of positive cells in epithelial dysplasia and squamous cell carcinoma lesions were significantly higher compared with those in control lesions (Figure 3).

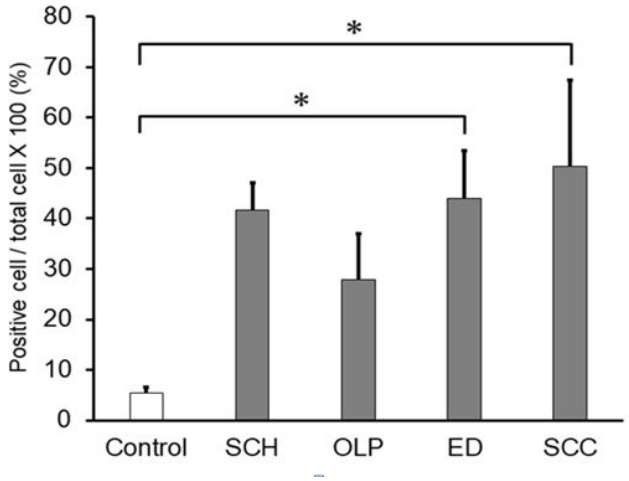

Figure 3: Percentage of positive cells out of total cells in each group determined by immunohistochemical staining with hexanoyl-lysine antibody. SCH=Squamous Cell Hyperplasia; OLP=Oral Lichen Planus; ED=Epithelial Dysplasia; SCC=Squamous Cell Carcinoma; *=$p<0.05$.

Discussion and Conclusion

Quantitation of protein carbonyls in the oral keratotic lesions revealed no correlation with age. This suggested that the cause of enhanced protein carbonylation in the lesions was unrelated to aging. Moreover, this study identified some interesting carbonylated proteins. Alpha-actinin-1 is an actin-binding protein. Fasciculation of actin is a change necessary for cell motility, which is a factor of cancer metastasis and invasion, and actin-4 is considered an actin-fasciculating protein. Enhanced expression of this protein is positively correlated with the outcome of infiltrating ductal carcinoma, lymph node metastasis of colorectal cancer, and some types of infiltration of oral squamous cell carcinoma [6]. Studies on tumor rejection antigen (gp96)1, which is a heat shock protein, have suggested it is a negative biomarker of squamous cell carcinoma, and an effective vaccine therapy based on this finding is expected [7]. Because protein carbonylation may be involved in the development or inhibition of these cancers, it is very interesting to investigate its potential role in inducing oral keratotic lesions, which is a precancerous state.

In the field of dermatology, a high level of carbonylated proteins in the stratum corneum of inflamed skin has been observed [8]. These studies focused on the outermost layer of the skin, the region constantly exposed to chronic inflammation, similar to the oral cavity. In addition, one study suggested that L-lysine could prevent protein carbonylation [1]. Although the physiological structure is different between the skin and the oral mucosa, if lipid peroxides are detected in, and contribute to causing oral mucosal keratotic lesions, the findings may lead to the establishment of a method inhibiting their production, and development of clinically applicable oral care products. Because subjects with a history of smoking and drinking were excluded from the current study, our findings may be useful not only in preventing oral mucosal keratotic lesions but also in designing clinical studies on reducing harmful carbonyls and utilizing redox therapy from an anti-aging medical point of view.

Localization of HEL in atherosclerotic lesions has been demonstrated immunologically, suggesting an association between HEL expression and disease. Accordingly, many studies on diseases accompanied by HEL expression have been performed [9]. Considering the influence of oxidative stress on blood vessel lesions, the results of our study may be an effective means to elucidate the development of oral mucosal keratotic lesions. Recently, studies on the association between protein carbonyls and lipid peroxidation have been reported [10]. The results of our study showing increased HEL expression strongly suggest the presence of local oxidative stress-induced lipid peroxides and may have influenced the production of protein carbonyls.

Establishment of a new treatment method for oral mucosal keratotic lesions, approached from the anti-aging point of view, can be expected based on the results of this study. Overall, the contribution to preventive medicine by connecting the current results to prevention and development of new therapies may be significant.

Conflict of Interest Statement

The authors have no conflict of interest relevant to this article.

Acknowledgment

A part of this research was supported by JSPS KAKENHI Grant-in-Aid for Scientific Research (C) Number 24593003.

References

1. Iwai I, Kuwahara T, Hirao T (2008) Decrease in the skin transparency induced by protein carbonylation in the stratum corneum. J Soc Cosmet Chem Jap 42: 16-21.
2. Van der Meji EH, Van der Waal I (2003) Lack of clinicopathologic correlation in the diagnosis of oral lichen planus based on the presently available diagnostic criteria and suggestion for modification. J Oral Pathol Med 32: 507-512.
3. Mohammed SA, Taqwa EO (2011) Detection of cytomorphological changes in oral mucosa among alcoholics and cigarette smokers. Oman Med J 26: 349-352.
4. Levin RL, Garland D, Oliver CN, Amici A, Climent I, et al. (1990) Determination of carbonyl content of oxidatively modified proteins. Methods Enzymol 186: 464-478.
5. Kato Y, Miyake Y, Yamamoto K, Shimomura Y, Ochi H, et al. (2000) Preparation of a monoclonal antibody to N (epsilon)-(Hexanoyl) lysine: application to the evaluation of protective effects of flavonoid supplementation against exercise-induced oxidative stress in rat skeletal muscle. Biochem Biophys Res Commun 274: 389-393.
6. Yamada S, Yamamoto S, Yoshida H, Yoshitomi I, Kawasaki G, et al. (2010) RNAi-mediated down-regulation of alpha-actinin-4 decreases invasion potential in oral squamous cell carcinoma. Int J Oral Maxillofac Surg 39: 61-67.
7. Akutsu Y, Matsubara H, Kano M, Usui A, Yoneyama Y, et al. (2011) Correlation between gp96 expression and the surgical outcome in patients with esophageal squamous cell carcinoma. Ann Surg Oncol 18: 832-837.
8. Iwai I, Shimadzu K, Kobayashi Y, Hirano T, Etou T (2010) Increased carbonyl protein level in the stratum corneum of inflammatory skin disorders: A non-invasive approach. J Dermatol 37: 693-698.
9. Ekuni D, Tomofuji T, Sande T, Irie K, Azuma T, et al. (2009) Periodontitis-induced lipid peroxide in rat descending aorta is involved in the initiation of Atherosclerosis. J Periodontal Res 44: 434-442.
10. Milic I, Melo T, Domingues MR, Domingues P, Fedorova M (2015) Heterogeneity of peptide adducts with carbonylated lipid peroxidation products. J Mass Spectrom 50: 603-612.