Original Article

Age- and sun exposure-dependent differences in 8-hydroxy-2'-deoxyguanosine and *N*$^{\varepsilon}$-(carboxymethyl)lysine in human epidermis

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Aging and exposure to sunlight are two major factors in the deterioration of skin function. In this study, thirty-six fixed human skin samples from sun-exposed and unexposed areas from young and old individuals were used to evaluate the localization of oxidative stress according to levels and distribution of 8-hydroxy-2'-deoxyguanosine and *N*$^{\varepsilon}$-(carboxymethyl)lysine in samples using immunohistochemistry. In the epidermis of the young, negligible amounts of 8-hydroxy-2'-deoxyguanosine and *N*$^{\varepsilon}$-(carboxymethyl)lysine were detected in unexposed areas, whereas nuclear 8-hydroxy-2'-deoxyguanosine and cytoplasmic *N*$^{\varepsilon}$-(carboxymethyl)lysine were increased in the lower epidermis in sun-exposed areas. In contrast, the aged presented prominent nuclear 8-hydroxy-2'-deoxyguanosine and nuclear *N*$^{\varepsilon}$-(carboxymethyl)lysine in the epidermis of unexposed areas, concomitant with dermal increase in *N*$^{\varepsilon}$-(carboxymethyl)lysine. However, the immunostaining of 8-hydroxy-2'-deoxyguanosine and *N*$^{\varepsilon}$-(carboxymethyl)lysine revealed a decrease in the epidermis of sun-exposed areas in the aged. These results suggest an age-dependent difference in the adaptation and protective mechanisms of the epidermis against sunlight-associated oxidative stress, thus necessitating distinct standards for evaluation in each age group. Further investigation is warranted to elucidate underlying molecular mechanisms.

***Key Words*:** **skin aging, UV, oxidative stress, advanced glycation end product**

Aging and exposure to sunlight are two major common factors in the deterioration of skin function. Ultraviolet (UV) radiation is a threat to human skin, causing photoaging and skin cancer. There is recent evidence that UV-induced skin damage is mediated by reactive oxygen species (ROS),[1,2] which target virtually all biomolecules. Alterations in genomic DNA are closely associated with carcinogenesis, making it an important target. Proteins are important in the skin as structural components for the protection of the underlying cells.[3,4]

8-Hydroxy-2'-deoxyguanosine (8-OHdG), one of the major oxidatively modified DNA bases, is induced by hydroxyl radical, singlet oxygen, photodynamic reaction or peroxynitrite, and is mutagenic when present in DNA replication.[5,6] 8-OHdG is most frequently used as a marker for evaluating oxidative stress. Previously, we reported an increase in the amount of epidermal 8-OHdG after single UV exposure at high doses,[7] and after chronic repeated exposure at low doses[8] in mice. We also applied 8-OHdG detection in a human skin-equivalent model.[9] Furthermore, *OGG1* knockout mice, lacking a repair enzyme for 8-OHdG present in the genome, were shown to be susceptible to UV-induced skin carcinogenesis.[10] The levels of 8-OHdG were shown to increase in rat skin by aging with high performance liquid chromatography using an electrochemical detector.[11]

N$^{\varepsilon}$-(carboxymethyl)lysine (CML) is an advanced glycation end product. It was originally thought to be important only in diabetic conditions, and increases have been reported in dermal collagen.[12–14] Interestingly, CML in skin collagen can predict the risk of future 10-year progression of diabetic retinopathy and nephropathy.[15] However, it recently became clear that many reactive oxygen species (ROS), including $^{\bullet}$OH,[16] peroxynitrite[17] and hypochlorous acid,[18] can ultimately generate CML. A highly specific monoclonal antibody to CML was recently produced, allowing for immunological methods of detection in various tissue types.[19] However, data from human samples and the localization of 8-OHdG and CML, especially in the epidermis, are lacking.

In the present study, we performed a pilot experiment to evaluate whether aging and sun-exposure alter the basal levels of 8-OHdG and CML in human skin.

Materials and Methods

Human samples. Formalin-fixed paraffin-embedded sections from skin samples, taken for diagnostic or therapeutic purposes, were used for the evaluation (Table 1). Permission was obtained from each patient or his or her representative, and the non-pathologic portion (e.g., non-tumorous tissue) was used for analysis. The ethical committees of Kinki University School of Medicine and Nagoya University Graduate School of Medicine approved the experiments.

Immunohistochemistry. Immunohistochemistry of 8-OHdG was performed using clone N45.1 (Nikken Seil, Fukuroi, Shizuoka, Japan; 20 μg/ml) and the avidin biotin complex method as previously described. Nuclear counterstaining was not performed.[20] Immunohistochemistry of CML was performed using clone 2G11 (Nippi, Toride, Ibaraki, Japan; 10 μg/ml) and avidin biotin complex method.[19] Both immunostaining procedures were preceded by a standard antigen retrieval method with immunosaver (Nissin EM, Tokyo, Japan). Immunostaining of the epidermis was scored independently as negative, weakly positive, moderately positive or intensely positive by two registered pathologists (S.T. and Y.Y.). Hematoxylin and eosin staining was also performed to obtain additional information.

Statistical analysis. The results were analyzed using analysis

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Table 1. Clinical data of human skin samples

Young-Unexposed		Young-Exposed		Aged-Unexposed		Aged-Exposed	
Age/sex	Diagnosis	Age/sex	Diagnosis	Age/sex	Diagnosis	Age/sex	Diagnosis
25M	Pemphigoid	29F	Intradermal nevus	56M	NS	95F	Bowen's disease
24M	Epidermal cyst	22M	NS	58F	Epidermal cyst	72M	Solar keratosis
26F	Dermatofibroma	22M	NS	54F	Compound nevus	90M	Bowen's disease
13M	Hemangioma	26M	Keloid	67F	Epidermal cyst	67M	Solar keratosis
18F	NS	27M	Intradermal nevus	55M	Epidermal cyst	61F	Solar keratosis
17F	NS	21F	Calcifying epithelioma	76M	Epidermal cyst	73F	Solar keratosis
15M	Epidermal cyst	2M	Keloid	70F	Epidermal cyst	52M	Solar keratosis
10F	Intradermal nevus	9F	NS	81F	Granulation	89F	Squamous cell carcinoma
8M	NS	18M	Intradermal nevus	67M	Epidermal cyst	87M	Solar keratosis
Means ± SEM: 17.33 ± 2.19		Means ± SEM: 19.56 ± 2.94		Means ± SEM: 64.89 ± 3.25		Means ± SEM: 76.22 ± 4.94	

Young denotes 2–29 years of age whereas aged denotes 52–95 years of age.
The age of unexposed and exposed groups in both the young and aged was not statistically different.
Unexposed samples are skin fragments either from chest, back or buttocks; all the exposed samples are from face.
Note that unaffected areas of skin regarding the main pathologic lesion were used for the present analysis.
M, male; F, female; NS, not specified.

of variance and Student's t test. $p<0.05$ was considered to be statistically significant.

Results

Skin histology. In the epidermis of areas unexposed to sun in the young, numerous wrinkles and dermal papillae were observed. The skin histology was not significantly different between the unexposed and exposed in the young (Fig. 1 A and D). The epidermis of the aged showed hyperkeratosis (Fig. 1 G and J, arrows). Dermal papillae became flattened in the aged, especially after repeated sun exposure. Dermal solar elastosis was prominent after persistent sun exposure over many years (Fig. 1 G and J, asterisks).

8-Hydroxy2'-deooxyguanosine (8-OHdG). The levels of 8-OHdG were low in the epidermis of the unexposed areas in the young (Fig. 1B), but were increased in most cases in the sun-exposed skin (Fig. 1E and Fig. 2). In contrast, the levels of 8-OHdG were significantly higher in the epidermis of the unexposed areas in the aged (Fig. 1H and Fig. 2). Furthermore, they were significantly decreased in the sun-exposed skin (Fig. 1K and Fig. 2). In some sun-exposed areas in the aged, sebaceous (Fig. 1N) and sweat (data not shown) glands presented intense nuclear immunostaining.

***N*$^{\varepsilon}$-(carboxymethyl)lysine (CML).** CML levels were low in the epidermis of the unexposed in the young (Fig. 1C), but significantly increased in the sun-exposed skin. CML was found mainly in the cytoplasm (Fig. 1F and Fig. 2). In contrast, CML levels were significantly higher in the epidermis of the unexposed in the aged and found primarily in the nuclei. Dermal fibroblasts and collagen fibers also exhibited strong positivity (Fig. 1I arrowheads, and Fig. 2). Of note was that CML levels decreased in the sun-exposed skin of the aged (Fig. 1L and Fig. 2). In some samples of the sun-exposed areas, sebaceous (Fig. 1M) and sweat (Fig. 1O) glands presented moderate immunostaining. Immunostaining was also increased in areas with dermal solar elastosis (Fig. 1L).

Discussion

This study for the first time evaluated the levels of 8-OHdG and CML simultaneously in human skin samples of the young and the aged. The results indicate steady levels of production and removal of both of the products. In human samples, the history and condition of each skin sample is much more complicated when compared to animal experiments.[8,21] Differences in age and persistent UV irradiation via sunlight revealed significant results.

Accumulation of 8-OHdG was prominent in the exposed skin in the young, but there were individual-to-individual differences that resulted in marginal statistical significance (Fig. 2A). Aging increased the basal levels of epidermal 8-OHdG as expected. This is likely due to the decrease in enzymatic repair rather than increased production. 8-OHdG levels in the epidermis of the aged were significantly lower in the sun-exposed skin, which was an unexpected result. Two possibilities are considered; 1) excessive oxidative stress caused 8-OHdG to be further modified into different products such as formamidopyrimidine-guanine,[22,23] or 2) the metabolism of epidermal cells were significantly lowered in the aged and sun-exposed. The nuclei of sebaceous and sweat glands, which were generally not immunostained in the other groups, were moderately stained, indicating the presence of oxidative stress in the skin of the aged and sun-exposed, thus supporting the former theory.

Immunohistochemical study of CML revealed the same tendency as 8-OHdG regarding aging and sun exposure (Fig. 2). The distinct difference was the intracellular localization in the epidermal cells. 8-OHdG was constantly observed in the nuclei. However, CML was present mainly in the cytoplasm in the young sun-exposed, and primarily in the nuclei in the aged unexposed epidermis (Fig. 1 F and I). Since CML is a lysine derivative, it can be present as CML-modified proteins. The results suggest that certain nuclear proteins are vulnerable to this modification or that the modified proteins have difficulty being removed from nuclei, as lysine is a key amino acid in histone modification for transcriptional regulation.[24] Further study is necessary to understand the significance of nuclear staining of CML. Presence of CML in fibroblasts and collagen fibers (Fig. 1I)[12–14] and in cases of solar elastosis (Fig. 1L),[25] are consistent with reported results. However, because the previous immunohistochemical study used frozen sections instead of paraffin-embedded sections,[25] detailed evaluation was not performed for the epidermis.

In conclusion, we evaluated the oxidative stress by immunostaining 8-OHdG and CML in human epidermis for the first time. There was a distinct difference in the distribution of both products as a result of aging and sun exposure. These factors should be considered when developing procedures to prevent photoaging and carcinogenesis.

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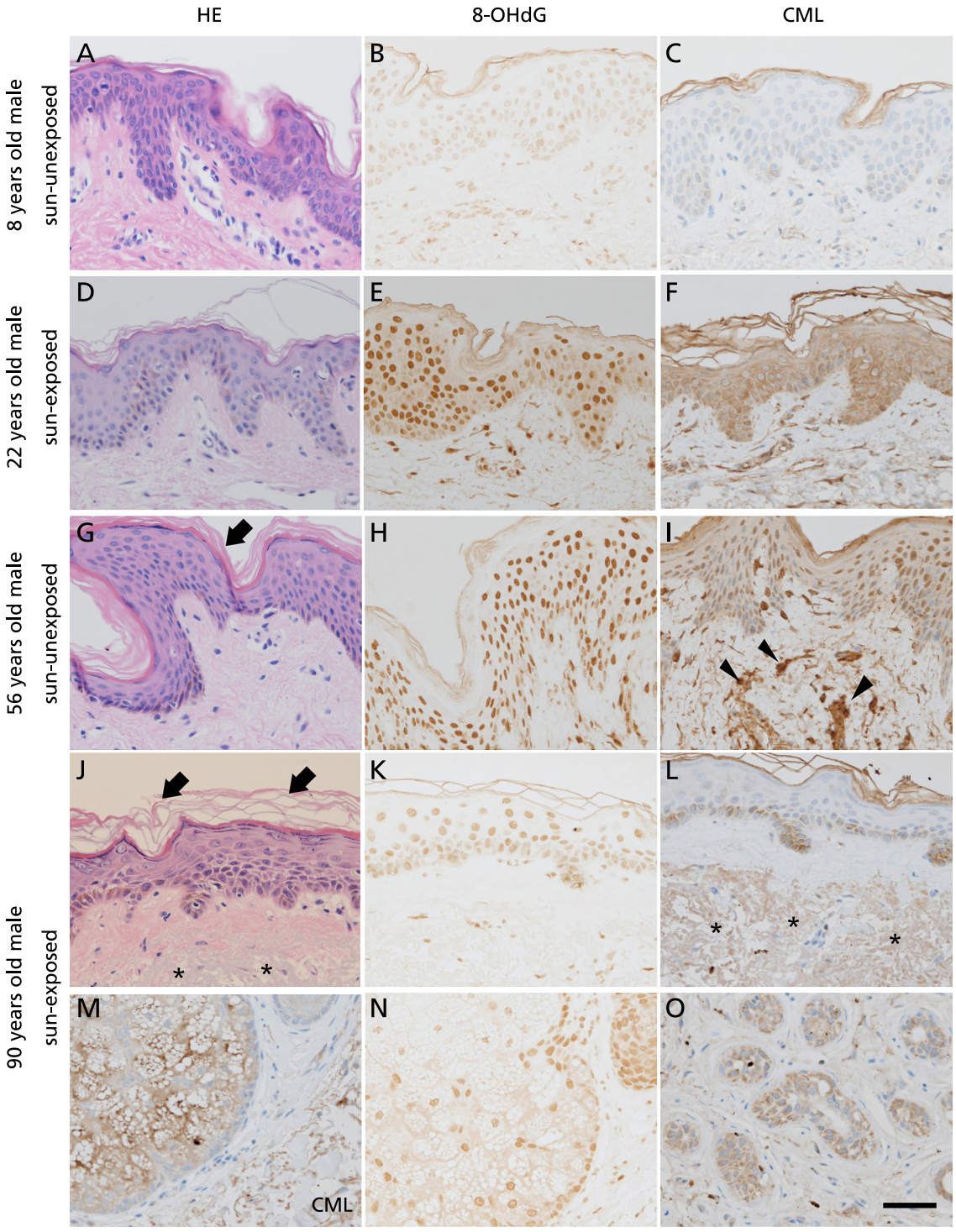

Fig. 1. Immunohistochemical analysis of oxidative stress in human skin of different ages and sites. Representative results are shown. Refer to text for details. HE, hematoxylin and eosin; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; CML, N^{ε}-(carboxymethyl)lysine. Arrows, hyperkeratosis; arrowheads, dermal fibroblasts and collagen fibers; *solar elastosis. Bar = 100 μm.

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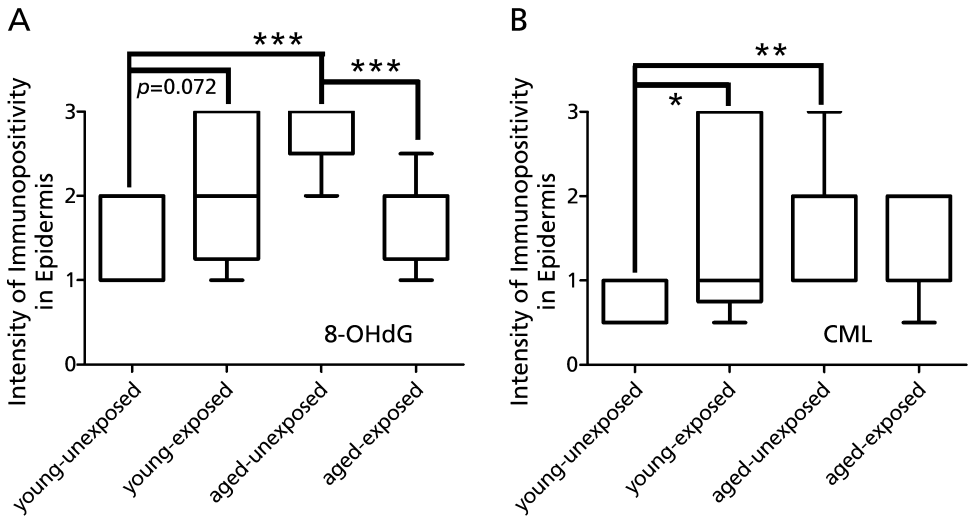

Fig. 2. Intensity of immunopositivity of (A) 8-hydroxy-2'-deoxyguanosine (8-OHdG) and (B) N^{ε}-(carboxymethyl)lysine (CML). Refer to text for details. Analysis of variance: A, $p<0.0001$; B, $p = 0.0226$. *$p<0.05$, **$p<0.01$, ***$p<0.0001$. $N = 9$ for each group. Refer to Table 1 for sample information.

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