Sacran, a natural polysaccharide, functions to defend the skin against air pollutants

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ABSTRACT

Sacran, a large molecular weight polysaccharide isolated from algae, is composed of 11 kinds of saccharides, including sulfate and carboxylic acid groups. Because of its unique structure, sacran can hold water at a five times higher capacity compared with hyaluronic acid and can form a gel-like sheet in the presence of 1,3-butylene glycol. The results of our previous study suggested that sacran can work as an artificial barrier against external stimuli such as air pollutants that increase the stress on humans. Thus, we examined whether sacran is able to reduce skin damage caused by tobacco smoke or exhaust gas as representative air pollutants focusing on oxidative stress. Sacran showed a higher affinity to corneocytes and reduced protein carbonylation by corneocytes exposed to tobacco smoke or exhaust gas. Further, sacran significantly improved the adverse effects initiated by tobacco smoke or exhaust gas in HaCaT keratinocytes, suppressing the decrease in cell viability, and reducing levels of intracellular reactive oxygen species and carbonylated proteins. Furthermore, topical application of a serum containing 0.04% sacran resulted in significantly reducing levels of carbonylated proteins in the stratum corneum of subjects who smoke tobacco. These results indicate that sacran reduces oxidative stress in or on the skin due to its interference with the contact of air pollutants.

INTRODUCTION

Recently, the influence of various air pollutants on human health has become an increasing concern around the world, especially in China, South Korea and Western Japan. In fact, the number of people who suffer from sensitive skin in Japan increases every year. In general, it has been demonstrated that polycyclic aromatic hydrocarbons (PAHs), which are the main components of air pollutants, induce inflammation by activating the aryl hydrocarbon receptor signaling pathway [1]. In that process, the roles of the over-expression of cytochrome P4501A1 and the excessive generation of reactive oxygen species (ROS) have been clarified [1].

In daily life, we are frequently exposed to tobacco smoke and exhaust gas as representative air pollutants. There is a risk that aldehyde compounds, which are present in tobacco smoke and in exhaust gas, causes the carbonylation of proteins in the skin. Our previous study demonstrated that ROS are generated from carbonylated proteins (CPs) when irradiated with blue light, which suggests that the accumulation of CPs is related to the generation of ROS [2]. Because air pollutants initiate skin problems after penetration into the skin, it is considered that it is very important to find out how to interfere with or prevent the penetration of air pollutants, PAHs and aldehyde compounds, into the skin. Therefore, skin care products that stimulate and/or reinforce skin barrier functions are strongly desired.

Sacran, a large molecular weight polysaccharide isolated from Aphanothece sacrum (Suizenji-nori) algae, has unique characteristics and is composed of 11 kinds of monosaccharides, including ionized ones containing sulfate groups and carboxylic acid groups. We have previously reported that sacran forms a hydrophobic gel-like film in combination with polyols and improves skin conditions in individuals who suffer from atopic dermatitis [3-4]. From those results, we expected that sacran would protect the skin against environmental stimuli such as chemicals and air pollutants (e.g. exhaust gas and tobacco smoke) due to its formation of an artificial skin barrier.

Thus, the purpose of this study was to clarify whether sacran reduces skin damage caused by air pollutants (tobacco smoke and exhaust gas) focusing on oxidative stress.
MATERIAL & METHODS

Materials

Sacran was extracted from an algae “Aphanothece sacrum” (Suizenji-anori) and was purified. Hyaluronic acid (HA) was purchased from Nacalai Tesque (Kyoto, Japan), and 1,3-butylene glycol (BG) was obtained from Kokyu Alcohol Kogyo Co. (Tokyo, Japan). Dulbecco’s modified Eagle medium (DMEM) and Hanks’ Balanced Salt solution with Ca²⁺ and Mg²⁺ (HBSS) were obtained from Nissui Pharmacy (Tokyo, Japan) and fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA, USA). The BCA Protein Assay Reagent kit was purchased from Pierce Chemical Co. (Rockford, IL, USA). LabCyte EPI-MODEL, reconstructed human epidermal equivalents (RHEEs) at 12 days, and their assay medium were obtained from Japan Tissue Engineering (Aichi, Japan). 6-[6-(Biotinylamino)hexanoylamino]hexanoylhydrazine (biotin-AC5-hydrazide) and Hoechst33342 solution were obtained from Dojindo Laboratories (Kumamoto, Japan). DyLight650-labeled streptavidin was obtained from Thermo Fisher Scientific (Waltham, MA USA). Rhodamine B (Basic Violet 10), NBD-Hydrazine (4-hydrazino-7-nitrobenzofurazanHydrazine), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Fluorescein-5-thiosemicarbazide (FTSC) and 2’,7’-dichlorodihydrofluorescein diacetate (H₂DCFDA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Source of tobacco smoke and collection of exhaust gas

Seven Stars (tar: 14 mg, nicotine 1.2 mg, Japan Tobacco) was used a source of tobacco smoke and exhaust gas was collected from a diesel car in a sealed bag.

Sacran aqueous solution

We used a 0.05% sacran aqueous solution and a 0.05% sacran aqueous solution including 10% BG (sacran/BG) for experiments as noted in the text. For comparison, we also used a 0.05% HA aqueous solution and 0.05% HA aqueous solution including 10% BG (HA/BG) where noted. In the text after this, polysaccharides denote sacran and HA.

Biotin conjugation to polysaccharides

Polysaccharides were reacted with 2 mg/mL biotin-(AC5)2-hydrazide in the presence of 20 mg/mL 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride and pyridine for 5 h. Biotin-conjugated polysaccharides were purified by dialysis against de-ionized water.

Dynamics of the penetration of polysaccharides into RHEEs

RHEEs were treated topically with biotin-conjugated polysaccharides and then were cultured for 24 h at 37°C. The supernatant of the culture medium was collected to quantify biotin-conjugated polysaccharides that penetrated through the RHEEs. Each RHEE was divided into two pieces, one used for histological examination and the other used to determine biotin-conjugated polysaccharides remaining in the RHEE. The dynamics of biotin-conjugated polysaccharides to penetrate into RHEEs were visualized by staining frozen thin sections with DyLight650-labeled streptavidin, and Hoechst33342 for nuclear staining. Fluorescence images were taken with a Thermo Fisher Scientific Inc. (MA, USA). RHEEs were homogenized in 500 μL PBS (pH 7.4) at 2700 rpm for 10 min with a μT-12 bead crusher (Taitec Corp., Saitama, Japan). After centrifugation, supernatants were used to determine biotin-conjugated polysaccharides in RHEEs with an ELISA method.
Transfer of corneocytes to glass slides

Corneocytes were collected by the tape-stripping method using cellophane tape (Nichiban Co., Ltd., Tokyo, Japan). Each piece of tape was tightly adhered to a glass slide using a 6 mm diameter piece to transfer corneocytes. The glass slides were immersed in xylene twice and then in EtOH to remove the cellophane by dissolving the adhesive agent.

Absorption of polysaccharides on corneocytes

Polysaccharide aqueous solutions were applied to corneocytes transferred to glass slides for 30 min at room temperature. After rinsing and drying, corneocytes were immersed in 1.0×10^{-3}% rhodamine B aqueous solution in the dark for 30 min. After rinsing, images were obtained with the Floid Cell Imaging Station. The staining level of rhodamine B in corneocytes was quantified by image analysis using a unique software, the Corneocytometry (CIEL Co., Ltd., Tokyo, Japan).

Interference of polysaccharides with protein carbonylation in corneocytes exposed to tobacco smoke or exhaust gas

Corneocytes treated with polysaccharide aqueous solutions were placed in a box filled with tobacco smoke or exhaust gas for 2 h and were incubated for 24 h at 37°C. Corneocytes were immersed in a 0.1 M MES-Na solution (pH 5.5) containing 20 µM FTSC for 1 h at 25°C in the dark. After rinsing, fluorescence images were taken with the Floid Cell Imaging Station. CP levels were quantified by image analysis using the Corneocytometry.

Preparation of polysaccharide-treated filters

Polysaccharide-treated filters were prepared as follows: A piece of membrane filter (0.45 μm JH, Merck Millipore) was soaked in 1 ml of polysaccharide aqueous solution and was then dried at 50°C.

Filtering effects of polysaccharide-treated filters on aldehyde compounds

Aldehyde compounds in DMEM bubbled with tobacco or exhaust gas were quantified with 25 μM NBD-Hydrazine in the presence of 0.05% trifluoroacetic acid. After incubation for 30 min in the dark, the fluorescence intensity (Ex; 470 nm, Em; 550 nm) was measured using a Spark 10M fluorescence microplate reader (Tecan, Männedorf, Switzerland).

Cell culture

HaCaT keratinocytes were cultured in DMEM with 5% FBS at 37°C in a humidified atmosphere containing 5% CO₂.

Influence of tobacco smoke or exhaust gas on HaCaT keratinocytes

Tobacco smoke or exhaust gas was bubbled in DMEM while it was stirred with a magnet stirrer (shown schematically in Fig. 1). HaCaT keratinocytes were cultured in DMEM treated with tobacco smoke or exhaust gas for 24 h. Cell viability as well as levels of intracellular ROS and CPs were measured as follows: Cell viability was measured using the neutral red assay. The intracellular ROS level was determined by the fluorescence intensity of oxidized H_2DCFDA per μg protein in each cell lysate. Intracellular CP levels were estimated by fluorescence intensity of FTSC labeling.

![Fig. 1](image-url) Method for treatment of DMEM by tobacco or exhaust gas.
Human use test
A serum containing 0.04% sacran and 15% polyols, including BG and pentylene glycol where noted, was used as a sacran serum. Four volunteers who were tobacco smokers were enrolled in the test. After explaining the aim of the test, formal consent was taken from each volunteer before the use test. The Sacran serum was topically applied on their cheeks, fingers, backs of the hands and lips twice a day, morning and night, for 4 weeks. Corneocytes were collected by the tape-stripping method and CP levels in the stratum corneum were quantified by image analysis using the Corneocytometry.

RESULTS
Dynamics of polysaccharides by topical application on penetration into RHEEs
In a previous study, we reported that sacran forms a gel-like sheet in the presence of BG. Therefore, we examined the potentials of polysaccharides to penetrate into RHEEs in the presence or absence of BG. In that examination, we used hyaluronic acid (HA) as a representative polysaccharide. Regardless of the presence or absence of BG, a higher fluorescence originated from sacran and from HA at the surface of the stratum corneum of RHEEs (Table 1). In addition, the results of quantification showed that more than 99% sacran or HA remained in the RHEEs regardless of the presence or absence of BG (Table 1). These results indicate that most polysaccharides topically applied on the RHEEs remained at the skin surface.

Absorption of polysaccharides on corneocytes
Corneocytes treated with sacran in the presence or absence of BG had reduced absorption of rhodamine B compared with those treated with HA (Fig. 2). In addition, sacran in combination with BG had a more extensively reduced absorption level of rhodamine B compared with sacran alone (Fig. 2). These results indicate that sacran has a higher affinity against corneocytes compared with HA.

Interference of polysaccharides on protein carbonylation in corneocytes exposed to tobacco smoke or exhaust gas
Corneocytes exposed to tobacco smoke or exhaust gas exhibited the presence of CPs at a higher incidence. Because previous examinations showed a higher affinity of sacran on corneocytes, it was expected to interfere with protein carbonylation caused by tobacco smoke or by exhaust gas. In that examination, sacran-treated corneocytes showed lower levels of CPs than HA-treated corneocytes. There was no significant difference between the presence or absence of BG (Fig. 3a, b).

Influence of tobacco smoke or exhaust gas on HaCaT keratinocytes
We evaluated the protective effects of polysaccharides against adverse effects on HaCaT keratinocytes caused by tobacco smoke or exhaust gas as representative air pollutants. Sacran showed a superior protection

Table 1 Penetration of biotin-conjugated sacran and HA with or without BG in RHEEs at 24 h after treatment.

<table>
<thead>
<tr>
<th></th>
<th>PBS HA</th>
<th>HA/BG</th>
<th>Sacran</th>
<th>Sacran/BG</th>
</tr>
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<tbody>
<tr>
<td>in reservoir solution</td>
<td>1.0±0.01(%)</td>
<td>1.0±0.01(%)</td>
<td>1.0±0.01(%)</td>
<td>1.0±0.01(%)</td>
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<tr>
<td>in RHEE model</td>
<td>98.0±0.02(%)</td>
<td>99.5±0.04(%)</td>
<td>99.5±0.03(%)</td>
<td>99.8±0.04(%)</td>
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against cell damage, intracellular levels of ROS and intracellular CPs compared with HA regardless of the presence or absence of BG (Fig. 4, 5, 6). Aldehyde compounds in DMEM were reduced by filtering through a sacran- or HA-treated filter (Fig. 7). Sacran was superior for its filtering effects against aldehyde compounds compared with

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**Fig. 2** Comparison of interactions with sacran or HA with or without BG by corneocytes.
(a) Stainability of rhodamine on tape-stripped corneocytes from human skin after treatment with sacran or HA with or without BG, Wilcoxon rank sum test, **p<0.01, ***p<0.001. (b) Representative images of rhodamine in corneocytes with each treatment (scale bar, 100 µm).

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**Fig. 3** Suppressive effects of sacran or HA with or without BG interacting with corneocytes on protein carbonylation by tobacco or exhaust gas.
(a) Changes in protein carbonylation in tape-stripped corneocytes from human skin after treatment with sacran or HA with or without BG, Wilcoxon rank sum test, **p<0.01, ***p<0.001. (b) Representative images of CPs in corneocytes after each treatment (scale bar, 100 µm). Control (-) denotes non-treated corneocytes and control (+) denotes corneocytes treated with tobacco or exhaust gas.

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HA (Fig. 7).

**Fig. 4** Suppressive effects of sacran or HA with or without BG on the cytotoxicity of HaCaT keratinocytes treated with tobacco or exhaust gas. Changes in cell viability of HaCaT keratinocytes after treatment with sacran or HA with or without BG, Wilcoxon rank sum test, **p<0.01, ***p<0.001. Control (-) denotes non-treated HaCaT keratinocytes and control (+) denotes HaCaT keratinocytes treated with tobacco or exhaust gas.

**Fig. 5** Suppressive effects of sacran or HA with or without BG on ROS generation by HaCaT keratinocytes treated with tobacco or exhaust gas. Changes in ROS generation in HaCaT keratinocytes after treatment with sacran or HA with or without BG, Wilcoxon rank sum test, **p<0.01, ***p<0.001. Control (-) denotes non-treated HaCaT keratinocytes and control (+) denotes HaCaT keratinocytes treated with tobacco or exhaust gas.

**Fig. 6** Suppressive effects of sacran or HA with or without BG on protein carbonylation by HaCaT keratinocytes treated with tobacco or exhaust gas. (a) Changes in protein carbonylation by HaCaT keratinocytes after treatment with sacran or HA with or without BG, Wilcoxon rank sum test, **p<0.01, ***p<0.001. (b) Representative images of CPs in HaCaT keratinocytes after each treatment (scale bar, 100 μm). Control (-) denotes non-treated HaCaT keratinocytes and control (+) denotes HaCaT keratinocytes treated with tobacco or exhaust gas.
Human use test

Topical application of the sacran serum to subjects who have a habit of tobacco smoking, resulted in significantly lower levels of CPs in the stratum corneum at all skin sites tested (Fig. 8).

DISCUSSION

In a previous study, we identified the unique characteristics of sacran that result in the formation of a gel-like sheet in the presence of polyols. That gel-like sheet suppresses water evaporation and penetration by chemicals [3]. These unique characteristics were expected to provide protection of the skin against external stimuli and water evaporation.

In fact, it has been reported that application of a sacran solution improves itching and facial rashes in atopic dermatitis patients based on a questionnaire survey [4]. In addition, sacran shows an improvement of the maturation status of corneocytes in healthy volunteers who had a history of atopic dermatitis. It is expected that the artificial barrier function of sacran is responsible for its effects in the human study. Thus, sacran is also expected to have

Fig. 7 Removal effects of sacran or HA treated filters on aldehyde compounds in DMEM by tobacco or exhaust gas. Changes in aldehyde compounds in DMEM after treatment with sacran or HA with or without BG, Wilcoxon rank sum test, **p<0.01, ***p<0.001. Control (-) denotes non-treated DMEM and control (+) denotes DMEM treated with tobacco or exhaust gas.

Fig. 8 Effects of sacran serum on protein carbonylation in corneocytes of subjects who smoke tobacco. (a) Changes in protein carbonylation in corneocytes after application of samples (pre: baseline of testing sites; post: after 4 weeks’ sample application), Wilcoxon rank sum test, **p<0.01, ***p<0.001. (b) Representative images of CPs in corneocytes at baseline and after sample application (scale bar, 100 µm).
protective effects against air pollutants. In order to characterize the potential effects of sacran against air pollutants, examinations using HaCaT keratinocytes in culture and a human use test were conducted focusing on oxidative stress induced by tobacco smoke or exhaust gas as representative air pollutants. In this study, to evaluate the effects of sacran, the effects of HA were compared as a representative polysaccharide. At first, to clarify the localization of sacran on/in the skin, a histochemical examination of topically polysaccharide-treated RHEEs was performed. Because sacran was observed on the surface of RHEEs, it was expected that sacran has an effect as an artificial barrier (Table 1). In subsequent examinations, we confirmed its higher affinity for corneocytes (Fig. 2a, b) and its superior filtering effects on CPs in corneocytes (Fig. 3a, b) and in HaCaT keratinocytes (Fig. 4, 5, 6). The filtering effects of sacran were clarified in a chemical examination measuring aldehyde compounds in DMEM (Fig. 7). In addition, the effects of sacran were higher than those of HA. In a previous study, HA failed to form a gel-like sheet in combination with BG. Furthermore, long term application of the sacran serum reduced CP levels in corneocytes of tobacco smokers. The sum of these results suggest that the unique physical characteristics of sacran are responsible for protection against adverse effects on the skin that are initiated by air pollutants.

CONCLUSION

We conclude from the results of this study that sacran protects against adverse effects initiated by exposure to tobacco smoke or exhaust gas. These results suggest the protective potential of sacran against oxidative stress induced by air pollutants. These findings strongly support the development of skin care products and make-up cosmetics formulated on sacran to protect the skin.

REFERENCES